

Regulation of *Fgf10* Gene Expression in the Prostate

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Declaration

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is part of it being concurrently submitted in candidature for any other degree.

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Abstract

Fibroblast growth factor 10 (FGF10) is a mesenchymal paracrine-acting factor that stimulates epithelial growth and is involved in the development of several branched organs, including the lungs, lachrymal glands and prostate. During branching morphogenesis in the lung, FGF10 is expressed in discrete areas of mesenchyme juxtaposed to branching epithelial tips. It has been proposed that paracrine factors produced in the epithelium and by differentiated stroma, such as sonic hedgehog (SHH) and transforming growth factor beta 1 (TGFbeta1) regulate the discrete expression pattern of FGF10. In the prostate, FGF10 expression is also confined to the mesenchyme that surrounds growing epithelial buds. In the prostate it has been proposed that FGF10 is involved in prostatic induction and epithelial branching morphogenesis. However, little is known about how *Fgf10* is regulated in the prostate, and the aim of this thesis was to investigate some of these regulatory mechanisms. This was done by developing a primary mesenchymal cell system in which to study *Fgf10* regulation; investigating how TGFbeta1 and testosterone affect *Fgf10* transcript expression in prostate cells and organs, and analysing the *Fgf10* promoter. In addition the effects of TGFbeta1 on prostate growth were assessed to determine if TGFbeta1 might have opposing effects to that of FGF10.

A primary stromal cell system, derived from the Ventral Mesenchymal Pad (VMP) was established and characterised. The VMP is a condensed area of mesenchyme found in both males and females that is required for prostatic induction in males, and is known to express *Fgf10*. After the first passage *in vitro*, primary VMP cells (VMPC) became larger and their growth rate slowed, suggesting that primary VMPC senesced after being plated out. VMPC maintained expression of *Fgf10*, *Tgfbeta1*, 2, and 3 transcripts at levels similar to those in the VMP *in vivo*. VMPC also expressed androgen receptor but did not show androgen responsive growth *in vitro*. It was concluded that primary VMPC were a good cellular system in which to study the regulation of *Fgf10* gene expression, and were used on their first passage.

It has been shown that TGFbeta1 and testosterone regulate *Fgf10* transcript expression in cells in culture. In studies presented here treatment of both cells and organs showed no direct regulation of *Fgf10* by testosterone. However, treatment of primary VMPC with TGFbeta1 reduced *Fgf10* expression fourteen-fold after 3 hours, but levels returned to control levels after 48 hours. A seven-hour TGFbeta1 treatment of VMP organ rudiments grown *in vitro* also decreased *Fgf10* transcript levels by three-fold, similar to the decrease in expression after seven hours in primary VMPC. Furthermore, regulation of *Fgf10* transcripts by TGFbeta1 was found to be specific for cells of the VMP and was not observed in urethral stroma. We next sought to extend our study into ventral prostate (VP) to determine if TGFbeta1 could regulate *Fgf10* transcript levels in the prostate. TGFbeta1 only decreased *Fgf10* transcript levels in the VP by approximately 1.5-fold, in contrast to VMP and VMPC that showed over a 3-fold repression. The reasons for the lower response of *Fgf10* to TGFbeta1 in VPs were not determined but may be due to epithelium inhibiting TGFbeta1 repression of *Fgf10*. This implies that factors present in the epithelium regulate the temporal and spatial expression of *Fgf10* in the prostate, similar to observations in the lung.

To further analyse *Fgf10* regulation, 6 kb of mouse genomic sequence 5' to the translation start, which was thought to contain the *Fgf10* promoter, was characterised. A transcription start site for *Fgf10* was mapped 704 nts 5' to the translation start site, by RNase protection assay. Comparisons of the mouse and human genomic sequences 5' to the *Fgf10* gene revealed several regions of high homology, suggestive of sites that control *Fgf10* gene expression. Deletion analysis of the *Fgf10* promoter identified a conserved element that mediated the majority of *Fgf10* promoter activity above basal core promoter activity, as well as mediating promoter downregulation by TGFbeta1. This element was located between nucleotides -50/-198, and contained a consensus Sp1 binding site. The promoter study provided further evidence that suggests TGFbeta1 regulates *Fgf10* gene expression in the prostate, as well as identifying a potential mechanism.

Next the effect of TGFbeta1 on VP development was characterised. The addition of TGFbeta1 to VPs inhibited 86% of testosterone-induced growth *in vitro* and

significantly increased numbers of epithelial-branched tips. After a six day TGFbeta1 treatment in the presence of testosterone, TGFbeta1 decreased proliferation of epithelial (67%) and stromal (70%) cells in the proximal to urethra region of the VP, but increased proliferation of epithelial (89%) and stromal (40%) cells in the distal to urethra region of the VP. This suggested that TGFbeta1 has different effects on proliferation depending on the location of the cells within the prostate and perhaps the level of cellular differentiation.

Previously it has been demonstrated that testosterone and TGFbetas regulate expression of *Tgfbeta* transcripts in adult prostates. To determine if the same effect was observed during development, VPs and VMPs were treated with testosterone and TGFbeta1 and *Tgfbeta* transcript levels were analysed by RPA. Testosterone and TGFbeta1 regulated *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* transcript levels in VPs and female VMPs cultured for three days, but the same effects were not observed over a six-day culture. As a previous study has shown that testosterone and TGFbeta1 did not affect *Tgfbeta* transcript levels in isolated prostatic stromal or epithelial cells it can be suggested that interactions between stroma and epithelium may be involved in the regulation of *Tgfbeta* transcript levels in prostatic rudiments.

Overall, I have provided an insight into the regulation of *Fgf10* and identified a possible mechanism involved in branching morphogenesis in the prostate. However, these data suggests that complex regulatory pathways, involving interactions between TGFbeta, testosterone and FGF10 are involved in regulating prostate morphogenesis.

Abbreviations

AP	alkaline Phosphatase
AR	androgen Receptor
bFGF	basic fibroblast growth factor (FGF2)
bp	base pairs
BrdU	5-bromo-2-deoxyuridine
°C	degrees Celsius
cDNA	copy DNA
CO ₂	carbon dioxide
CPSR	Controlled Process Serum Replacement – type 1
ddH ₂ O	double distilled water
DHT	dihydrotestosterone
DMEM	Dulbecco's Modern Eagle media
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphates
DPE	downstream promoter element
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene diaminetetra-acetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(b-aminoethyl ether)-n,n,
ER	endoplasmic reticulum

FCS	foetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FSH	follicular stimulating hormone
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLGAG	heparin-like glycosaminoglycan
HPRT	hypoxanthine guanine phosphoribosyl transferase
HSP	heat shock protein
IAA	iso-amyl alcohol
IgG	immunoglobulin
IL β	interleukin beta
Inr	Initiator element
Kb	Kilobases
KCl	potassium chloride
KDa	KiloDaltons
KGF	keratinocyte growth factor (FGF7)
LA	Luria Agar
LB	Luria Broth
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
MAPK	Mitogen-Activated-Protein-Kinase
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	message ribonucleic acid

NP40	nonidet P40
NTP	Nucleoside 5'-Triphosphate
ONPG	o-nitrophenyl β -D-galactopyranoside
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PIPES	Piperazine-N, N-bis[2-ethanesulfonic acid]: 1,4-Piperazinediethanesulfonic acid
PLC γ	Phospholipase C gamma
PMSF	Phenylmethanesulfonylfluoride
PS-1	Prostate stromal cells
PSA	Prostate specific antigen
RNA	ribonucleic acid
RNase	ribonuclease
RPA	RNase protection assay
Rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SM	smooth muscle
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TBS	tris buffer saline
TBST	tris-buffer saline tween 20
Tfm	testicular feminized mouse strain

TGF α	transforming growth factor alpha
TGFbeta	transforming growth factor beta
TRAMP	transgenic adenocarcinoma mouse prostate
UGE	urogenital epithelium
UGM	urogenital mesenchyme
UGS	urogenital stroma
UGT	urogenital tract
UR	urethra
URSC	urogenital stromal cells
³² P-UTP	uridine 5'-[α - ³² P]-triphosphate, triethylammonium salt
UV	ultraviolet
VMP	ventral mesenchymal pad
VMPC	ventral mesenchymal pad cells
w/v	weight to volume ratio
X-gal	5'-bromo-4'-chloro-3'-indolyl- β -D-galactopyranoside

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1 Introduction

Prostate development is dependent on androgens and mesenchymal-epithelial interactions (Chung et al., 1991; Cunha and Chung, 1981; Tenniswood, 1986). Androgens bind to pad of condensed mesenchymal cells (called the ventral mesenchymal pad, VMP) of the male urogenital sinus resulting in paracrine signalling that regulates epithelial budding and branching. Epithelial budding can also be induced in the female VMP in the presence of testosterone (Lasnitzki and Mizuno, 1977; Takeda et al., 1986; Timms et al., 1995). This suggests that the VMP expresses the necessary paracrine factors that regulate prostate development. A mesenchymal paracrine factor expressed in the male and female VMP is fibroblast growth factor 10 (FGF10). *Fgf10* transcripts are expressed during periods of prostatic growth suggesting that *Fgf10* plays an important role in prostate organogenesis (Thomson and Cunha, 1999). In fact FGF10 may be acting as a paracrine factor that regulates epithelial branching morphogenesis (Thomson and Cunha, 1999). FGF10 also plays a role in the branching morphogenesis and growth of many organs (table 1.1). As androgens act on the VMP to induce prostate development and *Fgf10* is expressed in the VMP it could be suggested that *Fgf10* may be regulated by testosterone. Testosterone has been shown to stimulate *Fgf10* expression in prostate cells (Lu et al., 1999) although some controversy surrounds this result (Thomson, 2001). Also in other systems, such as the lung, numerous factors have been shown to regulate *Fgf10* during branching morphogenesis (Bellusci et al., 1997b; Lebeche et al., 1999). Thus, as the regulation of *Fgf10* has not been thoroughly investigated in the prostate, the aim of this thesis was to investigate the regulation of *Fgf10* transcript levels during prostatic development.

This thesis has addressed the regulation of *Fgf10* gene expression in primary prostate cells and organ cultures. TGFbeta1 has been identified as a factor that regulates *Fgf10* transcript levels in these systems. Also the regulation of *Fgf10* transcription was investigated by promoter analysis. An Sp1 binding site was shown to mediate basal

levels of *Fgf10* transcription and TGFbeta1 appeared to repress *Fgf10* transcription through this site. *Tgfbeta* transcript isoforms have been implicated in prostate development and we have demonstrated that although TGFbeta1 inhibits prostate growth, it has differential effects on prostate cell proliferation. These studies have examined prostate development and this chapter will discuss prostate anatomy and function, followed by an in depth review of prostate development. Finally as the regulation of *Fgf10* transcript levels by TGFbeta1 was examined the next part of this chapter will discuss the role of FGFs and TGFbetas in prostate development.

1.1 The Prostate

1.1.1 The Adult Prostate

The prostate gland is found exclusively in mammals and functions by contributing secretory proteins and other factors to seminal fluid. In adult human males the prostate is a small oval shaped organ located surrounding the urethra at the base of the bladder. The structure of the human prostate lacks distinct lobular organization and has been studied extensively to be defined as three discrete morphological regions; the peripheral zone, the transition zone, and the central zone (Fig. 1.1 A) (McNeal, 1969; McNeal, 1988). Unlike human, the rodent prostate gland has distinct lobular organisation and is made up of four distinct lobes, the anterior, dorsal, lateral (and dorsolateral) and ventral (Fig. 1.1 B). There is no clear correlation between the structures found in the rodent prostate than those in the human prostate, although primarily descriptive evidence suggests the rodent dorsolateral lobe is most similar to the human peripheral zone (Abate-Shen and Shen, 2000). Also similar to the human prostate, these lobes are found circumventing the neck of the bladder and have characteristic features of ductal branching and secretory protein production (Hayashi et al., 1991; Sugimura et al., 1986a) but there is no evidence for different secretions in human zones.

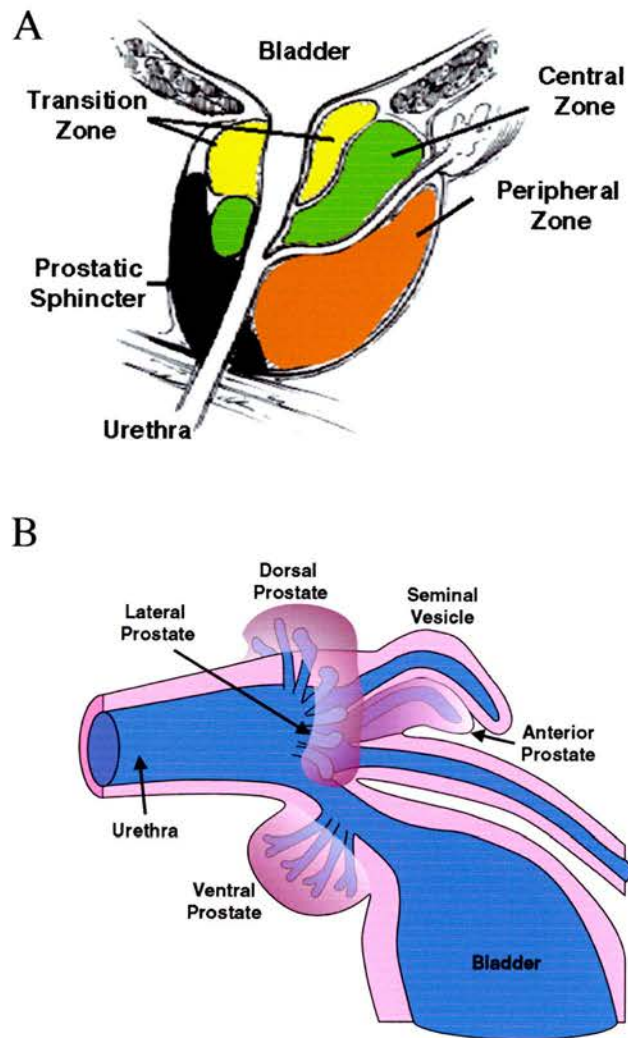


Fig. 1.1 Schematic illustration of the anatomy of the human (A) and rat (B) prostate (adapted from Abate-Shen 2000 and Thomson 2001, respectively).

The glandular component of the prostate is made up of at least three distinct cell types that differ in morphological appearance, functional significance and relative abundance (Fig. 1.2). The main glandular epithelial cell is the luminal cell. These cells are differentiated androgen-dependent cells that produce prostatic secreted proteins. Basal cells are the second key epithelial cell type found in the prostate and are located between luminal cells and the basement membrane, which surrounds the prostatic duct. Basal cells do not produce secretory proteins and might function to protect cells against DNA damage (Bui and Reiter, 1998; De Marzo et al., 1998). Interspersed between basal and luminal cells are neuroendocrine cells that are believed to support the growth of luminal cells by paracrine signalling (Abrahamsson, 1999; di Sant'Agnese, 1992; di Sant'Agnese, 1998). The glandular epithelial cells are juxtaposed with an underlying stromal compartment consisting of smooth muscle cells, fibroblasts, endothelial cells, nerves and extracellular matrix (ECM). Interactions between stromal and epithelial cells play an important role in maintaining prostate architecture and prostate function (Chung and Davies, 1996; Lee, 1996). In fact stromal cell paracrine signalling is involved in androgen induced growth of normal prostatic epithelial cells (Gao et al, 2001).

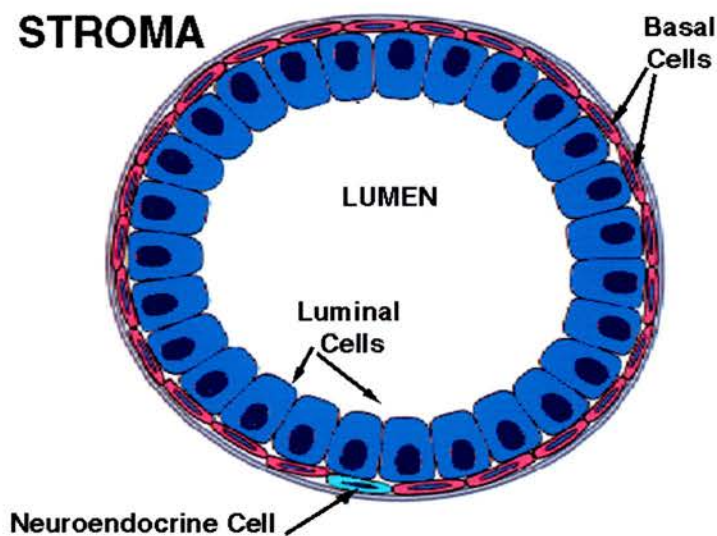


Fig. 1.2 Schematic illustration of the cell types within prostatic ducts (adapted from Abate-Shen 2000)

1.1.2 Development of the Rat Prostate

The development of the rat prostate is a complex process involving interactions between steroids and the urogenital sinus (UGS) tissue (Chung et al., 1991; Cunha and Chung, 1981; Tenniswood, 1986). Primitive UGS originates from the terminal hindgut, through the division of the cloaca by the urorectal septum during mid-gestation in rodents (this occurs earlier in humans). The bladder and the penile urethra form from the cranial and caudal ends of the primitive UGS, respectively. The prostate gland forms in the intermediate region. This region is referred to as the UGS and consists of urogenital mesenchyme (UGM) and urogenital epithelium (UGE). Development of the prostate requires the presence of androgens and signalling between UGM and UGE (Cunha and Donjacour, 1987). The rat urogenital structures can first be seen at e14, and first visually distinguished as bladder and urogenital tissue at e17 (Hayward et al., 1996a). The first signs of prostate formation in the rat were thought to occur when solid cords of epithelium from the UGE bud into the UGM at e19 (Fig. 1.3B) (Hayward et al., 1996a). However more recently, molecular insights into the earliest stages of prostate formation were derived from the analysis of the *Nkx3.1* homeobox gene in the mouse (Bhatia-Gaur et al., 1999; Sciavolino et al., 1997). The expression of *Nkx3.1* first appeared in the mouse UGE two days prior to the formation of prostatic lobes (Fig. 1.3A). On the day preceding the development of prostatic lobes *Nkx3.1* became more intense in the urethra epithelium that forms prostatic buds. When prostatic buds emerge, *Nkx3.1* expression became more restricted to the epithelium of all the outgrowing buds in the different prostatic lobes (Fig. 1.3C). Thus, expression of *Nkx3.1* transcripts may demarcate regions where prostatic buds will arise from the UGE.

During prostate organogenesis the epithelial buds grow at their tips (Sugimura et al., 1986b) and become highly branched. Numerous reports have demonstrated that epithelial bud induction and growth during prostate development are due to androgens acting via the mesenchyme (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980; Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985). These

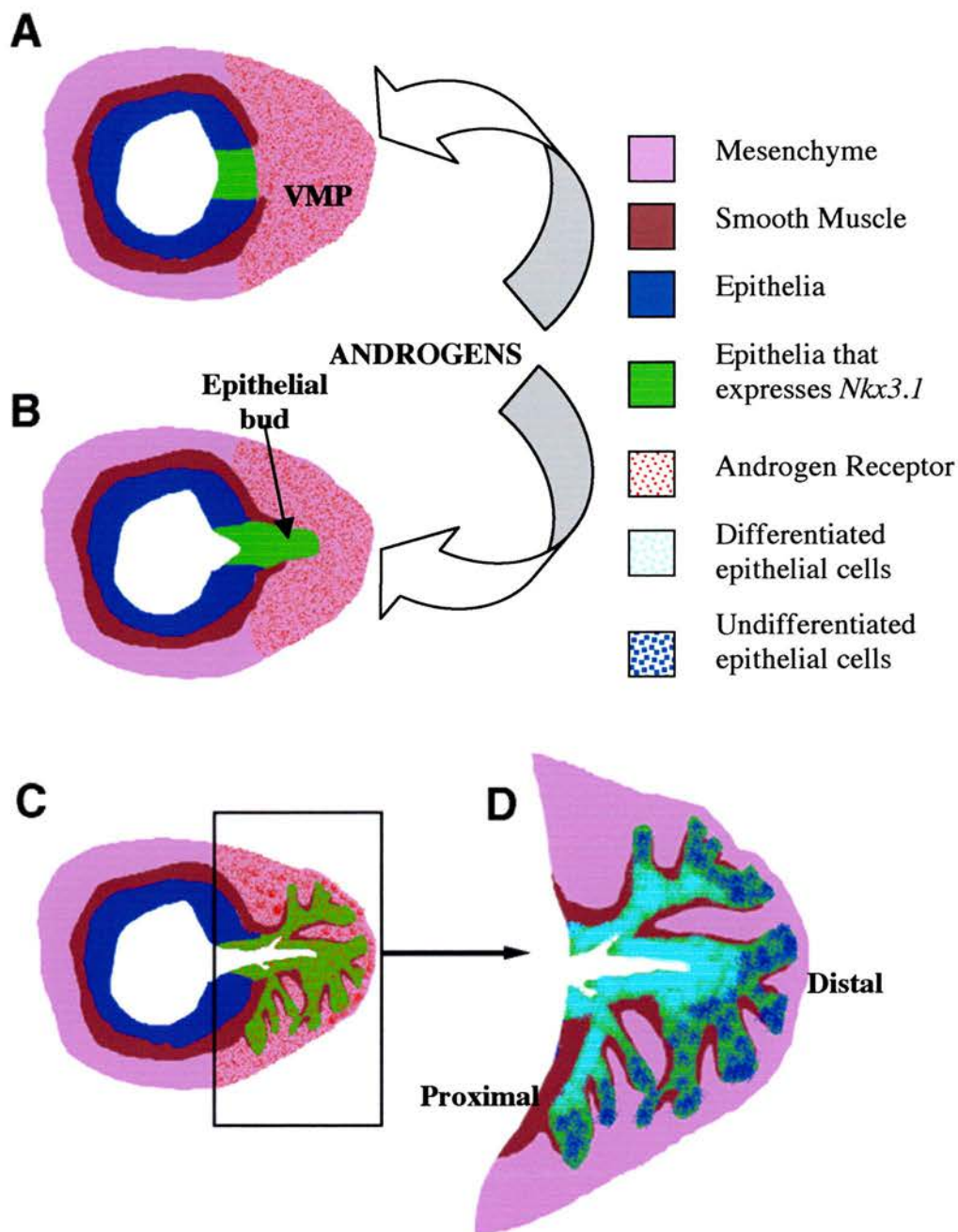


Fig. 1.3 Diagram showing the distribution of UGS tissue and factors involved in prostate morphogenesis. At e17 (A) the male UGS consists of UGE (blue), SM (brown), UGM (pink) and AR (red dots). The green regions represent UGE that expresses *Nkx3.1*. No epithelial budding has occurred and a SM gap (discussed later) is apparent. At e19 (B) the UGE that expressed *Nkx3.1* has budded through the SM gap into the VMP. Note the lack of AR in the epithelia, but AR is still expressed in the mesenchyme. At this stage androgens promote prostate development via interactions with the VMP. By P0 (C) the epithelia has branched into the mesenchyme and now expresses AR. Also mesenchyme adjacent to the epithelia has differentiated into SM expressing cells. On closer inspection (D) the SM runs along the epithelia branches but SM expression becomes less apparent towards the distal tip, and does not cover the tip. Also D demonstrates the distribution of differentiated epithelial cells. Differentiated cells are shown in light blue dots that are located in the proximal region and undifferentiated cells (dark blue spots) are shown in the distal regions.

reports have therefore postulated that androgens regulate paracrine factors produced by the mesenchyme that influence epithelial proliferation leading to prostatic bud formation and branching morphogenesis. Androgens also appear to regulate the differentiation of epithelial cells during prostate development. At birth, prostate epithelia uniformly express cytokeratins 5, 7, 8, 14, 18, and 19 (Hayward et al., 1996a). During development of the prostate and differentiation of the epithelia, cytokeratins segregate to the basal and luminal subtypes. In the rat prostate, this process occurs initially in the epithelium proximal to urethra proceeding towards the distal region (Fig. 1.3D). The adult prostate contains both luminal cells (expressing cytokeratins 8, 18, and 19) and basal cells (expressing cytokeratins 5, 7, 14, and 19). In adulthood, androgens have been postulated as being involved in maintaining prostatic differentiation and secretions (Cunha and Chung, 1981; Hayward et al., 1996a).

The patterns of branching morphogenesis in the prostate may be similar in mechanism to branching observed in other branched organs (Bellusci et al., 1997b; Mailleux et al., 2002). Numerous similarities between branching and expressed factors have been observed between the lung and the prostate. Fibroblast growth factor signalling in the lung has been extensively examined (Bellusci et al., 1997b; Peters et al., 1994) and FGF7 and FGF10 appear to be involved in both lung (Bellusci et al., 1997b; Post et al., 1996) and prostate development (Sugimura et al., 1996; Thomson and Cunha, 1999; Thomson et al., 1997). Figure 1.4 shows the factors that may be involved in prostate development of which some will be discussed now. *Fgf10* is expressed in the UGM around the elongating epithelial buds (Thomson and Cunha, 1999) and may be involved in regulating ductal branching. *Fgf7* is also expressed in the UGM (Finch et al., 1995a) and stimulates ductal branching (Sugimura et al., 1996). *Tgfbeta1* is expressed predominantly in the UGM and TGFbeta1 protein has been localised around the growing tips (Timme et al., 1994). The transcription factor *Gli* is expressed in the mesenchyme and has been associated with *Shh* signalling (Podlasek et al., 1999a). *Hox* genes are expressed in both the epithelial and mesenchymal compartments of the UGS and abnormalities in prostate development are observed in *Hox* gene mutations (Oefelein et

al., 1996; Podlasek et al., 1999b; Podlasek et al., 1999c). Other factors expressed in the epithelium include *Nkx3.1* and *Shh*. *Nkx3.1* gene expression is localised to emerging prostatic epithelial buds (Sciavolino et al., 1997) and defects in the *Nkx3.1* gene have been linked to prostatic epithelial hyperplasia and defects in branching morphogenesis (Bhatia-Gaur et al., 1999). *Shh* has been postulated as being involved in prostate development by regulating branching morphogenesis and ductal bud formation (Lamm et al., 2002; Podlasek et al., 1999a). As many of these factors play a role in development of the lung (Fig. 1.7) it is possible that there are numerous conserved mechanisms between prostatic and lung branching morphogenesis.

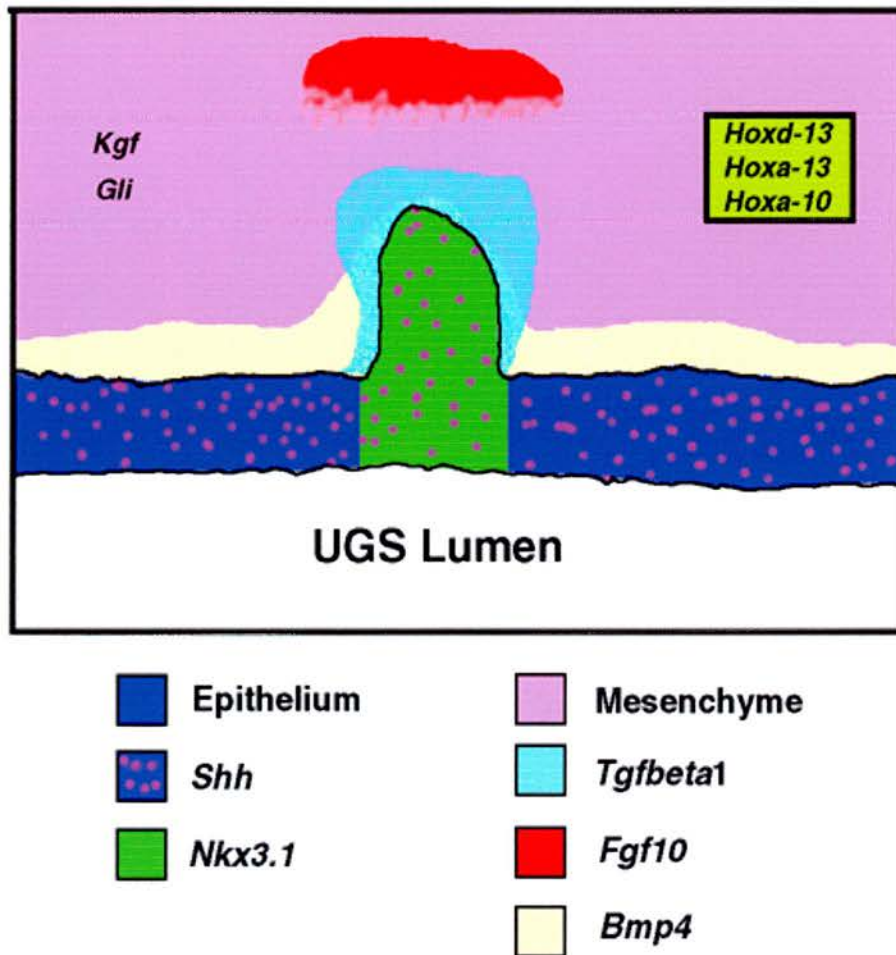


Fig 1.4 Factors that have been postulated as being involved in regulating budding and branching morphogenesis in the prostate. (Figure adapted from (Lamm et al., 2001))

We have addressed prostate development and briefly described the roles of androgens and cellular interactions in this process. So next I will discuss the endocrinology and mesenchymal-epithelial interactions of prostate development in more detail. Also factors postulated as being involved in prostate development and branching morphogenesis, such as FGF10 and TGFbeta1, will be discussed.

1.1.3 Endocrinology of Prostate Induction and Growth

The major steroids involved in development of the prostate are androgens. Androgens are C_{19} steroids characterised by their ability to stimulate and maintain the development of secondary sexual characteristics. Testosterone is the principal androgen and is mainly secreted by androgen producing cells in the testis; Leydig cells. The testis is an endocrine gland where androgen secretion is primarily driven by luteinizing hormone (LH) and spermatogenesis by follicular stimulating hormone (FSH) produced in the pituitary (Fig. 1.5). LH and FSH secretion is under the control of LH releasing hormone (LHRH) secreted from the hypothalamus. Testosterone exerts a negative feedback regulation on LH and FSH production by the pituitary gland and paracrine regulation of Sertoli cell function. FSH and androgen stimulate inhibin production by sertoli cells that selectively suppresses FSH production (Fig. 1.5).

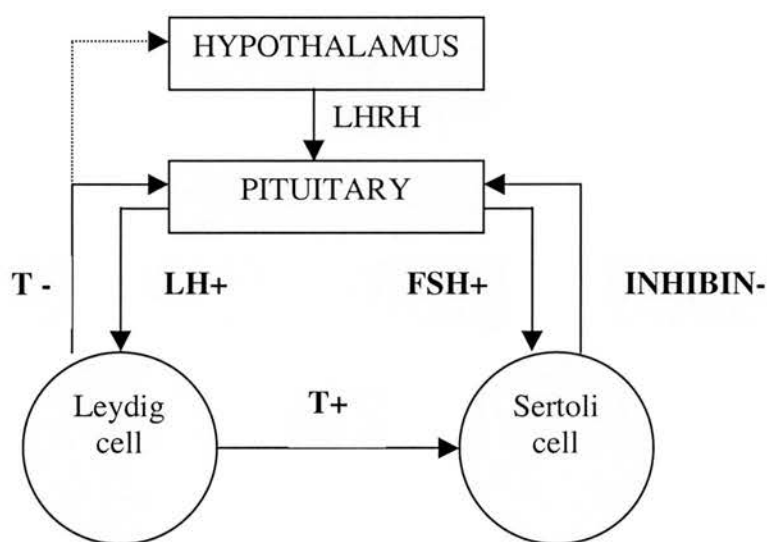


Fig. 1.5 The hypothalamic-pituitary-gonadal axis

Most secreted androgens (>98 %) circulate in the blood bound to albumin and steroid binding globulins. The steroid binding capacity of these proteins determines the

proportion of androgen that circulates freely. Only free testosterone is able to enter the cell and hence free testosterone is the biologically active form. More than 90% of testosterone in the cell is converted to 5 α -dihydrotestosterone (DHT) through the action of the enzyme 5 α -reductase. DHT binds at a higher affinity (five times) to the AR than testosterone and is a more potent androgen (Wilbert et al., 1983). Although DHT binds at a higher affinity to AR than testosterone data supports the idea that prostate development does not absolutely require DHT; androgens that cannot be converted to DHT will elicit the growth of prostatic rudiments *in vitro* (Foster and Cunha, 1999). Also, inhibition of 5 α -reductase does not completely impair prostate development (Imperato-McGinley et al., 1992).

The levels of circulating androgens correlate with prostate development and production of prostatic fluid during adulthood; androgen levels are relatively high towards the end of gestation, decrease one day after birth and increase again during puberty in rats (Corpechot et al., 1981). Hence androgens may be involved in numerous processes during prostate organogenesis. In fact during development, androgens may promote epithelial bud induction from the UGE into the UGM in the male. Androgens may induce UGE budding by acting on the UGM to instructively induce the morphogenesis and differentiation of adjacent UGE *in vivo* (described below) (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980; Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985). Also, administration of androgens to female urogenital tracts *in vitro* results in prostatic budding (Lasnitzki and Mizuno, 1977; Takeda et al., 1986). Surprisingly the formation of the VMP is independent of androgens, as the VMP is found in females, but it is clear that the VMP has the ability to induce prostate organogenesis in the presence of androgens (Timms et al., 1995). This might suggest that paracrine factors are secreted by the VMP in response to androgens, which regulate prostatic budding. Several growth factors that are produced by stromal cells in response to androgens have been identified and shown to influence prostatic epithelial cells (reviewed in Cunha et al., 1992). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF; FGF7), FGF10, transforming

growth factor α (TGF α) and insulin-like growth factors I and II can all stimulate growth of prostatic epithelial cells (Cohen et al., 1991; Culig et al., 1996; Itoh et al., 1998b; Marengo and Chung, 1994; Peehl and Rubin, 1995; Sugimura et al., 1996; Thomson and Cunha, 1999). However some controversy surrounds the effect of androgens on the regulation of some of these genes (Thomson, 2001), but there is no doubt that they positively regulate the proliferation of prostate epithelial cells. Also androgens have been shown to repress gene expression of factors that have inhibitory effects on prostatic epithelial and stromal cells, such as transforming growth factor beta (TGFbeta) (Itoh et al., 1998a; Kyprianou and Isaacs, 1989). Androgens are not only involved in epithelial bud induction but are also important in promoting epithelial differentiation (discussed later) (Cunha and Chung, 1981).

Another function of testosterone during development of the prostate may be its regulation of smooth muscle (SM) expression along the UGT. It has been reported that the position of the VMP coincides with a gap in the SM at the junction of the urethra and bladder (Thomson et al., 2002). The gap in SM was consistent in the male and female urogenital tracts from e17 to e19, after which a sexually dimorphic difference became apparent. After e19, the layer of SM was reported to be continuous in females, but an obvious gap in SM remained in the male (Fig. 1.3). The SM layer may act as a barrier to epithelial buds and thus prevent prostate development. Thus the closure of SM gap in females may prevent epithelial budding while maintenance of a gap in males provides an area that allows epithelial buds to grow into the VMP. Therefore this report suggests an alternative pathway that androgens can regulate development of the prostate. Hence androgens appear to have multifunctional roles in prostate organogenesis.

Other steroids potentially involved in prostate development are oestrogens. Exposure to low doses of oestrogens during mouse development have been reported to increase adult prostate weight and AR levels, as well as significantly increasing prostatic glandular budding (Nonneman et al., 1992; Timms et al., 1999; vom Saal et al., 1997). In contrast, higher doses of oestrogen administered to neonates have caused a permanent

suppression of prostate growth, a reduced response to androgens and increased epithelial and stromal hyperplasia (Naslund and Coffey, 1986; Prins, 1992; Singh and Handelsman, 1999; Turner et al., 1989). It has been shown that the reduction in response to androgens is due to a decrease in AR levels, and could possibly be linked to a reduction in circulating androgen (Prins, 1992). More recent research has suggested that the effect of oestrogens on development of the prostate may be due not only to a repression of the hypothalamic-pituitary-gonadal axis (Fig. 1.3), but also oestrogens may have a direct effect on prostate development (Jarred et al., 2000).

1.1.4 Stromal-Epithelial Interactions

1.1.4.1 Mesenchymal-Epithelial Interactions during Development

The role of mesenchymal-epithelial interactions in prostate formation has been extensively defined through tissue recombination studies, performed by Cunha and colleagues (Cunha, 1996; Cunha and Chung, 1981; Cunha et al., 1987; Cunha and Lung, 1978; Hayward et al., 1997). Tissue recombination experiments used dissection and enzymatic isolation of epithelium and mesenchyme from embryonic UGS and/or from other tissues. The tissues are recombined and transplanted into adult rodent male hosts. Tissue architecture and proteins normally secreted by the prostate were examined. Tissue recombination studies have led to the conclusion that prostatic differentiation requires the interaction of epithelia and mesenchyme. However, although a prostate will only form using mesenchyme from embryonic UGS, a wide range of epithelia of endodermal origin, including those from differentiated male or female adult tissue, can form prostates when recombined.

Tissue recombination experiments have been performed using tissue combinations from mutant animals. In particular, recombination experiments using Testicular-feminized (*Tfm*) mutant mouse strains have been thoroughly examined. The *Tfm* mouse strain

carries a defective AR that results in premature termination of AR synthesis and loss of function of androgen signalling (He et al., 1991). As would be expected male *Tfm* mice have no prostate and recombination experiments of epithelial and mesenchyme from *Tfm* mice with wildtype examined the role of AR in prostatic development (Fig. 1.6) (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980). Prostatic structures appeared when *Tfm* epithelium was recombined with wild-type mesenchyme suggesting that AR in the mesenchyme was necessary for prostate development. Prostatic structures did not form when wild-type epithelium was recombined with *Tfm* mesenchyme, suggesting that expression of AR only in the epithelium was not enough to induce prostatic formation. Overall these results determined that androgens act on the mesenchyme of developing prostates to induce prostate development and that AR expression in the epithelium is not required to initiate prostatic budding. However, although *Tfm* UGE combined with wild-type mesenchyme forms prostatic ducts, the epithelium lacked production of prostatic secretory proteins. This suggested that following development androgens act on the epithelium to promote prostatic secretory proteins. The conclusions derived from *Tfm* recombination experiments correlated with studies examining the expression pattern of AR (Hayward et al., 1996a; Hayward et al., 1996b) and autoradiographic studies of androgen binding sites (Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985) in the developing prostate. AR expression was restricted to the mesenchyme preceding and during initial stages of prostatic budding. After prostatic buds have formed and begun to develop, the epithelium expresses AR. This suggests that an androgen-induced mesenchymal paracrine factor regulates epithelial budding and androgen-regulated factors are not produced in the epithelium. However the function of AR in epithelial cells is most likely to mediate prostatic secretions and epithelial differentiation.

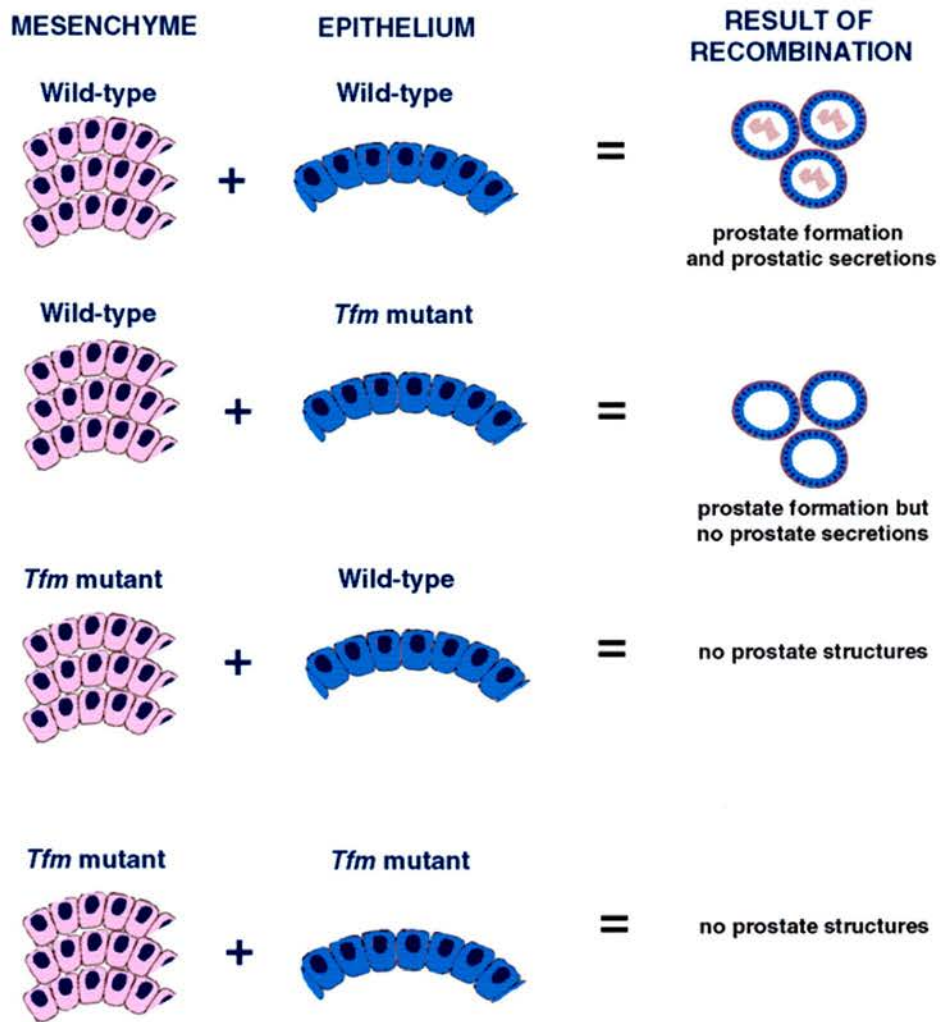


Fig. 1.6 depicting results from recombination experiments performed by Cunha *et al.* 1981, using *Tfm* and wild type mice tissues.

Signalling can also occur from the epithelium to the mesenchyme. As the epithelial bud grows into the VMP it becomes obvious that cellular changes occur in the mesenchyme (Hayward *et al.*, 1996b). Mesenchyme cells start expressing smooth muscle α actin (a smooth muscle marker) around the periphery of the epithelial bud. The expression of SM α actin is followed by vinculin, myosin, desmin, and laminin in an orderly sequence in a distal to proximal manner along the prostatic ducts (Hayward *et al.*, 1996b). Also by using tissue recombination techniques it was demonstrated that different prostatic

epithelium elicit different patterns of stromal development (Hayward et al., 1998). Tissue recombination experiments consisting of adult human prostatic epithelium and rat UGM, grafted beneath the renal capsule of a rodent host, revealed that the rat mesenchyme differentiated into thick sheets of smooth muscle, characteristic of the human and not the rat prostate (Hayward et al., 1998). Also a solid cord of human epithelium grew into the surrounding mesenchyme and canalised expressing markers for basal and luminal cells. Hence epithelial to mesenchyme signalling is most likely to regulate the differentiation of the mesenchyme into smooth muscle cells and the reciprocal interactions regulate epithelial growth and differentiation.

1.1.4.2 Stromal-Epithelial Interactions during Adulthood

A similar mesenchymal-epithelial interaction that occurs during prostatic organogenesis also occurs during adulthood between smooth muscle and epithelial cells. It has been hypothesised that adult prostatic smooth muscle maintains epithelial structure and function and, in a reciprocal fashion, prostatic epithelium maintains smooth muscle differentiation (Hayward et al., 1996c). It is likely that aberrant interactions between the epithelium and smooth muscle play an important role in prostate carcinoma and although neoplastic changes occur in the epithelium compartment, the role of the stromal compartment has been relatively neglected. However it has been demonstrated that smooth muscle failed to differentiate in tissue recombinants of neoplastic prostatic epithelium and UGM (Hayward et al., 1996c) demonstrating that neoplastic epithelium regulates the pattern of SM expression. Also tissue recombination experiments have suggested that paracrine signalling from the stromal compartment may play an important role in cancer progression (Hayward et al., 1997; Olumi et al., 1999). The paracrine signals that mediate stromal-epithelial interactions in prostate carcinoma have not yet been identified, but may include, members of the FGF family or TGFbeta family (Cunha, 1996; Djakiew, 2000). Members of these growth factor families have been investigated in development, not only in the prostate but also in other systems. Some of these factors thought to be important during prostate development and that have been

examined in this thesis will be discussed next, not only in the context of prostate biology but also their role in development of similar branched organs such as the lung, and disease.

1.2 Fibroblast Growth Factors in the Development of the Prostate

Numerous FGFs have been identified and the family contains at least 23 proteins. FGFs induce mitogenic, chemotactic, and angiogenic activity in cells derived from mesodermal and neuroectodermal origins (reviewed in (Basilico and Moscatelli, 1992)). The FGF family members play key roles in the development of several organs including the limbs, lung, brain, and prostate (reviewed in (Ornitz and Itoh, 2001; Powers et al., 2000)). FGFs are involved in developmental processes from the earliest time points of embryogenesis to after birth. In fact there are probably very few organs in which FGFs are not involved in some developmental role. FGF gene expression and function has been extensively examined in both limb and lung organogenesis (Bellusci et al., 1997b; Lebeche et al., 1999; Ohuchi et al., 1999; Ohuchi et al., 1997; Yonei-Tamura et al., 1999) and FGFs serve as an essential regulator of their development (Min et al., 1998; Sekine et al., 1999). It is therefore no surprise that FGFs function in the development of the prostate (Lu et al., 1999; Nemeth et al., 1998; Thomson and Cunha, 1999; Thomson et al., 1997), as key processes have been shown to be similar between lung and prostate formation (Lamm et al., 2001; Lebeche et al., 1999). FGF7 (KGF) and FGF10 play important roles in the development of the prostate (Thomson and Cunha, 1999; Thomson et al., 1997) and it remains to be seen if other FGFs e.g. FGF8 or FGF9 are involved in regulation of prostatic growth. However as this thesis has examined the regulation of *Fgf10* gene expression, FGF10 will be discussed in more detail than any other FGF. Also it is important to discuss the role that FGF10 plays in lung formation since more research has been performed on these processes than in prostate

development. However initially a general overview of the role that FGF10 plays in development will be discussed.

1.2.1 FGF10

The *Fgf10* gene was first isolated from rat embryos by homology-based polymerase chain reaction (Yamasaki et al., 1996). Yamasaki *et al.* discovered that the expression pattern of *Fgf10* mRNA was quite different from other FGFs. *Fgf10* transcript expression was more restricted than other members of the FGF family and was predominantly expressed in the rat embryo and adult lung. However no function was described for the protein. The next reported *Fgf10* gene isolation was cloned from mouse tissue (Beer et al., 1997). Beer *et al.* investigated the function of *Fgf10* by examining the characteristics of the FGF10 protein, the expression pattern of *Fgf10* transcripts in isolated tissues and during wound repair, and also the regulation of *Fgf10* transcript expression in fibroblast cells. It was shown that FGF10 is most homologous to FGF7 (57 % identity at the amino acid level). FGF10 was predicted to encode a protein with a predicted mass of 23.6 kDa (Beer et al., 1997). The FGF10 protein was also predicted to express a leader sequence necessary for ER-Golgi secretion. FGF7 also has a leader sequence (Finch et al., 1989) and is efficiently secreted from cells (Brauchle et al., 1994; Chedid et al., 1994). Beer *et al.* demonstrated that the FGF10 protein was not in the media suggesting the mouse FGF10 protein could be either inside the cell, associated with cell surface, or associated with the ECM. It was hypothesised that the mouse FGF10 protein enters the secretory pathway but remains associated with either the cell or ECM for two reasons; 1) at least one form of the protein is N-glycosylated and glycosylation occurs in the ER-Golgi apparatus and not in the cytosol, and 2) heparin, which is known to compete for binding of FGFs to the ECM and cell surface proteoglycans (Burgess and Maciag, 1989), releases mouse FGF10 proteins into the media (Beer et al., 1997; Igarashi et al., 1998).

FGF7 and FGF10 have been shown to have similar paracrine mechanisms. FGF7 is predominantly expressed in mesenchymal cells (Finch et al., 1989), while the receptor for FGF7 is expressed in epithelial cells (Finch et al., 1989). FGF10 also acts in a paracrine manner as *Fgf10* transcripts have been detected in the mesenchyme of numerous organs (Bellusci et al., 1997b; Ohuchi et al., 1997; Thomson and Cunha, 1999) and its receptor (FGFR2 IIIb) in epithelium (Igarashi et al., 1998). It has also been demonstrated that FGF7 and FGF10 have similar receptor binding properties and target cell specificities during development (Igarashi et al., 1998).

Although FGF7 and FGF10 act on similar cells and are mainly expressed in same organs the regulation of *Fgf7* and *Fgf10* is vastly different. *Fgf7* transcripts were strongly up regulated by numerous cytokines (Brauchle et al., 1994; Chedid et al., 1994) but most of these factors did not have the same affect on *Fgf10* transcript levels (Beer et al., 1997). In fact TGF β 1 and TNF α both repressed the levels of *Fgf10* transcripts in 3T3 fibroblasts suggesting that *Fgf7* and *Fgf10* transcript levels could be oppositely regulated under conditions where high levels of these cytokines are expressed. FGF7 and FGF10 are also involved in lung development and were differentially regulated in that system (Lebeche et al., 1999). SHH, FGF1, and FGF2 appeared to stimulate expression of *Fgf7* transcripts, while FGF1 and FGF2 had no effect on *Fgf10* transcript levels but SHH decreased *Fgf10* gene expression. This suggests that although *Fgf7* and *Fgf10* have a high sequence homology and similar functions their gene expression is differentially regulated. Also Beer *et al.* (1997) demonstrated that *Fgf10* transcripts were rapidly decreased indicating a short half-life of the mRNA, typical features of highly regulated genes.

FGF7 and FGF10 are also regulated differently at their protein level (Igarashi et al., 1998). Members of the FGF family interact with ECM components after secretion and during receptor signalling (described below) (Flaumenhaft et al., 1990; Mansukhani et al., 1992; Moscatelli, 1987; Yayon et al., 1991). Important components of the ECM that have been shown to bind and regulate distribution of these proteins are heparin and

heparan-like glycosaminoglycans (HLGAGs). Heparin exerts differential effects on FGF10 and FGF7, by stimulating FGF10 and inhibiting FGF7 mitogenic activity (Igarashi et al., 1998). These effects could be one possible mechanism through which different responses to FGF10 and FGF7 may be mediated although they signal through the same receptor. Also FGF10 is glycosylated and remains predominantly cell or ECM associated (Beer et al., 1997), while FGF7, which is also glycosylated, is readily secreted into the medium of producing cells (Finch et al., 1989).

The original expression study performed by Beer *at al.* (1997) demonstrated the presence of *Fgf10* transcripts in various adult mouse tissues. *Fgf10* transcripts were mainly expressed in the lung, brain and dermis. More extensive studies on *Fgf10* transcript expression have been performed and have shown that FGF10 may play important roles in a variety of different systems (Table 1.1).

Table 1.1 Summary of the role of FGF10 in different processes

Organ System	The Role of FGF10	References
Lung	Localised sources of FGF10 in the mesoderm regulate endoderm proliferation and bud outgrowth	(Bellusci et al., 1997b; Min et al., 1998; Ohuchi et al., 2000; Sekine et al., 1999)
Limb	FGF10 is a mesenchymal factor involved in the initial budding as well as the continuous outgrowth of vertebrate limbs	(Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999; Yonei-Tamura et al., 1999)
Reproductive tract	FGF10 identified as an essential factor in prostate development, involved in bud outgrowth and branching morphogenesis. Also it has been shown to be important during development of the glans penis and glans clitoridis	(Lu et al., 1999; Nakano et al., 1999; Perriton et al., 2002; Thomson and Cunha, 1999)
Brain	The spatial expression of FGF10 in the brain indicates that it has a distinct role in the brain	(Hattori et al., 1997)
Lacrimal glands	FGF10s role in induction of lacrimal gland budding is to stimulate proliferation and, in turn, FGF10 combines with other factors to provide the instructive signals required for lacrimal gland development	(Makarenkova et al., 2000)
Kidney	FGF10 appeared to be important in ureteric bud branching and branch elongation, and thus may play a role in the determination of nephron	(Cancilla et al., 2001; Qiao et al., 2001)

	number and patterning of the kidneys	
Mammary	FGF10 important in mammary gland development	(Mailleux et al., 2002)
Thymus	FGF10 important in thymic epithelial signalling	(Revest et al., 2001)
Pancreas	FGF10 plays an essential role in the mesenchymal interaction required for the proper development of the pancreas	(Bhushan et al., 2001; Miralles et al., 1999; Yamaoka et al., 2002)
Heart	The arterial pole of the mouse heart forms from FGF10-expressing cells in pharyngeal mesoderm	(Kelly et al., 2001)
Colorectal cancer	Findings indicate that FGF10 and FGFR IIIb may contribute to the growth of colorectal cancer cells	(Matsuike et al., 2001)
Uterus	The <i>in vivo</i> spatial expression pattern suggests that FGF10 is a endometrial stromal cell-derived mediator of uterine epithelial function	(Chen et al., 2000)
Teeth	FGF10 suggested to be a survival factor that maintains the stem cell population in developing incisor germ cells in mice	(Harada et al., 2002)
Adipose tissue	FGF10 plays an important role in adipogenesis by regulating expression of factors by autocrine/paracrine mechanisms	(Sakaue et al., 2002; Yonei-Tamura et al., 1999)
Skin	FGF10 is required for embryonic epidermal morphogenesis in the skin but is not essential for hair follicle development. May be involved in wound healing	(Beer et al., 1997; Suzuki et al., 2000; Tagashira et al., 1997; Tao et al., 2002)
Ear	FGF10 signalling important for inner ear morphogenesis	(Pirvola et al., 2000)
Bladder	May have an important role in regulating growth, differentiation and repair of urothelium cells in the bladder	(Bagai et al., 2002)

1.2.2 FGF10 Signalling

Members of the FGF family have a central core of 140 amino acids, which fold into twelve antiparallel β -strands forming a cylindrical barrel and is highly homologous between different family members (reviewed in (Powers et al., 2000)). The amino- and carboxy-terminal stretches are more variable between members of the family. The amino terminus of most FGFs contains a leader sequence, including FGF10, and this sequence is involved in secretion (Blobel, 1980). The regions involved in receptor and heparin binding are located in the β -strands, and a defining feature of the FGF family is

a strong affinity for heparin and HLGAGs (Burgess and Maciag, 1989) of the ECM. This interaction between FGF and HLGAG may be involved in regulation of FGFR signalling (Plotnikov et al., 1999; Venkataraman et al., 1999) and thus FGF activity. In fact the interactions between FGFs and these factors may not only limit their diffusion and release into interstitial spaces but also stabilize FGFs against denaturation and proteolysis (Flaumenhaft et al., 1990; Moscatelli, 1987). Also cell surface HLGAGs are physiologically significant as they were originally shown to be required for the high affinity binding of FGFs to their receptors (Mansukhani et al., 1992; Yayon et al., 1991). The high affinity binding caused by the presence of HLGAGs mediates mitogenesis and angiogenesis caused by FGF signaling (Aviezer et al., 1994; Rapraeger et al., 1991). These results led to an hypothesis that HLGAGs not only mediates the recruitment of FGFs to the cell surface and stabilized FGFs against denaturation but also induced a change in the presentation of the FGFs or FGFR that promotes binding of the two (Klagsbrun and Baird, 1991). Research has been focused on the later hypothesis and it is now a common understanding that HLGAGs promote FGFs ability to activate receptors and that they induce receptor dimerization (Ornitz and Itoh, 2001; Ornitz et al., 1992; Powers et al., 2000; Spivak-Kroizman et al., 1994).

There are four different genes encoding FGF receptors (FGFR), designated FGFR1-4, which are responsible for FGF-mediated signal transduction (Johnson and Williams, 1993). These receptors are characterised by two or three immunoglobulin (Ig)-like domains in the extracellular region and a tyrosine kinase domain in the intracellular regions. Additional diversity of the FGFR family is generated by alternative splicing that results in differing ligand specificity (Chellaiah et al., 1994; Miki et al., 1992). FGF10 can act on two types of FGFR: FGFR1 IIIb and FGFR2 IIIb (Beer et al., 2000; Igarashi et al., 1998). Numerous FGFR signalling pathways have been identified (Goldschmidt-Clermont et al., 1991; Larsson et al., 1999; Mohammadi et al., 1996; Zhan et al., 1994). However signalling pathways of different FGFRs are very similar, which is probably due to the high degree of homology between the different receptor proteins (Johnson and Williams, 1993). Also this may be due to the fact that the principal

difference between FGFRs has been postulated as being in the level of tyrosine kinase activity and that this level is important in determining the response to ligand activation of the receptor (Raffioni et al., 1999).

One of the most characterised systems on which FGF10 plays a key role is during development of the lung. Next I will discuss the role that FGF10 plays in lung growth and development, as the processes of lung branching morphogenesis appear to be similar to that of the prostate.

1.2.3 The Role of FGF10 in Development of the Lung

Development of lungs in mice begins at e9.5 by lung buds emerging from each ventro-lateral side of the foregut endoderm. The buds then grow in a caudal direction away from the gut endoderm. The two primary buds elongate and undergo branching morphogenesis to give rise to five lobes, four on the right and one on the left. It has been shown that branching morphogenesis of the lung, as well as other organs, depends on epithelial-mesenchymal interactions that are mediated by a complex network of interactions including those of transcription factors, peptide growth factors, and their receptors (Fig. 1.7) (reviewed in (Cardoso, 2000; Cardoso, 2001)).

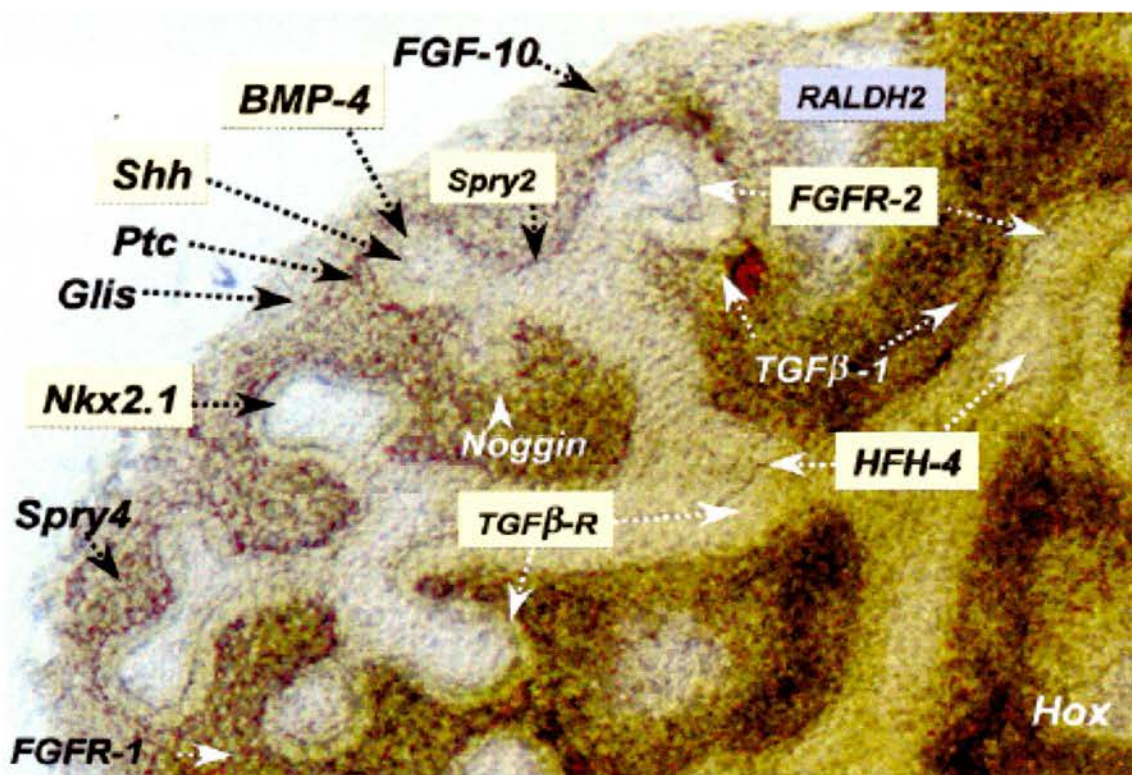


Fig. 1.7 The spatial distribution of gene expression in the developing lung. Epithelial expression is represented in yellow boxes and all other genes are expressed in the mesenchyme with exception of RALDH2 (blue box). RALDH2 is found in lung mesothelial cells and tracheal mesenchyme (taken from Cardoso, 2000).

New insights into development of the lung have been derived from other developing structures such as the limb or the respiratory system of the *Drosophila* (Klamt et al., 1992; Sutherland et al., 1996). There are key differences between the *Drosophila* respiratory system and mouse lung. In particular cells migrate in the *Drosophila* trachea system but cell proliferation promotes lung airway development. However factors involved in regulating the *Drosophila* respiratory system have proved to be very similar to those in the mouse lung. The *Drosophila* gene, *branchless* (*bnl*), is critical for patterning of tracheal branching (Sutherland et al., 1996). The secreted *bnl* binds to the *breathless* receptor (*btl*) on nearby tracheal cells, stimulating receptor signalling and promoting budding of primary branches. As each primary branch grows towards a patch of cells that express *bnl*, expression of *bnl* is inhibited. Occasionally expression of *bnl* turns on at a different site and the primary branch continues to grow towards the new

site. Hence *bnl* is important for the initial patterning of tracheal branching. The *bnl-btl* interactions are also important for secondary budding pattern and terminal branching. Mutant analyses of the *bnl* and *btl* genes have demonstrated the necessity of the ligand and receptor during tracheal development (Klamt et al., 1992; Sutherland et al., 1996).

Bnl is an *Fgf* homolog and a 99 amino acid stretch of *bnl* is 30 % - 40 % identical to several vertebrate *Fgfs* (Sutherland et al., 1996). The *bnl* mutant (Sutherland et al., 1996) has a similar phenotype to that of the *Fgf10* knockout mutant mice (Min et al., 1998; Sekine et al., 1999). *Fgf10* mutant mice demonstrated that FGF10 was required for the initial branching of the primordial buds during the early phase of embryonic lung development (Min et al., 1998; Sekine et al., 1999). Hence the function of *Fgf10* in mice and *bnl* in *Drosophila* are very similar in that both are critical for initial pulmonary branching events. FGF10 signals via FGFR2 IIIb (Igarashi et al., 1998) and this interaction was considered to be similar to the *bnl-btl* signalling processes in the *Drosophila* (Sutherland et al., 1996). In fact the initial breakthrough in identifying mesodermal factors that regulated branching morphogenesis controlling mammalian lung development initially came with the targeted expression of the dominant expression of *Fgfr2 IIIb* (Peters et al., 1994). FGFR2 IIIb is expressed in the endoderm of developing lungs (Cardoso, 2001) and numerous FGFs have been shown to signal via this receptor (Table 1.2). The dominant negative *Fgfr2 IIIb* completely blocked airway branching and epithelial differentiation, without prohibiting outgrowth, establishing a specific role for FGFs in branching morphogenesis in the mammalian lung (Peters et al., 1994). FGF10 appeared to be the most likely member of FGF family involved in this process, based on its expression and localisation within the lung mesenchyme (Bellusci et al., 1997a; Park et al., 1998).

Fgf10 transcripts have been shown to be mesenchymal-expressed in a localised fashion surrounding the epithelial buds (Bellusci et al., 1997b). *Fgf10* gene expression appears to precede formation of lung buds, and its expression is down regulated once the bud has formed (Bellusci et al., 1997b). Hence *Fgf10* gene expression is dynamically regulated

during lung organogenesis. If the mesenchyme is removed from the epithelial bud, recombinant FGF10 can substitute the effect caused by the mesenchyme by inducing generalised budding (Bellusci et al., 1997b; Park et al., 1998). This suggested that FGF10 produced by the mesenchyme was necessary for lung budding during development. However creating a gradient of FGF10 protein by implanting a heparin bead soaked in FGF10 caused the lung bud to grow towards and engulf the bead (Park et al., 1998; Weaver et al., 2000). Park *et al.* also grafted an FGF10-soaked bead into intact e11.5 lung explants, in an organ culture system, to produce similar results (Park et al., 1998). The FGF10-soaked bead appeared to augment endogenous FGF10 signalling causing bud growth to be redirected towards the bead. Also it was demonstrated that FGF10 acts over relatively short distances in lung explants and that the chemotactic effect of FGF10 was reduced (from 150 μm to between 50 to 75 μm) in lung explants with mesenchyme attached, compared to buds without mesenchyme (Park et al., 1998; Weaver et al., 2000).

1.2.4 Modulation of FGF10 Signalling in the Lung

The dynamic expression of *bnl* in *Drosophila* development and *Fgf10* in the mouse lung suggests that branching morphogenesis might require FGF10 levels to be precisely controlled by factors expressed in both the mesenchyme and epithelium. Numerous factors have been identified that might play important roles in regulating branching morphogenesis and therefore postulated as modulating FGF10 signalling in the lung. These include FGFs, sonic hedgehog (SHH), bone morphogenetic protein 4 (BMP4), and TGF β 1.

FGFs Numerous FGFs were identified as being expressed in development of the lung and shown to influence lung development by treatment of embryonic lungs in organ cultures. A complex regulatory network between FGF1, FGF2 and FGF7 has been proposed (Lebeche et al., 1999). However, FGF9 and FGF10 are the first known FGFs to be detected in the developing lung and research has suggested that FGF9 could

act as a potential regulator of FGF10, via activation of FGFR1 signalling in early lung development (Arman et al., 1999).

SHH *Shh* is expressed in the endoderm by e9.5 and expression is continued in the developing lung epithelium (Bellusci et al., 1996). As in FGF10 knockout mice, *Shh* expression is essential for branching morphogenesis but not for the initial budding of the foregut endoderm in the developing lung (Litingtung et al., 1998; Pepicelli et al., 1998). Also lung mesenchyme in *Shh* knockout mice diffusely expressed *Fgf10* contrasting with the restricted pattern of expression in wild-type mice (Pepicelli et al., 1998). This suggests that *Shh* not only inhibits *Fgf10* gene expression but it regulates the spatial distribution of FGF10 and this regulation is required for the proper patterning of the lungs during development. Other data supports this hypothesis as over-expression of *Shh* in transgenic mice increased mesenchymal proliferation and decreased expression of *Fgf10* (Bellusci et al., 1997a; Bellusci et al., 1997b).

BMP4 *Bmp4* is initially expressed in the foregut mesenchyme suggesting it plays a role in start of lung development (Weaver et al., 1999). During branching morphogenesis BMP4 is expressed in the epithelium of branching airways (Weaver et al., 2000). Increasing gradients of FGF10 in the mesenchyme induces levels of *Bmp4* expression during branching morphogenesis. Also as BMP4 inhibits epithelial cell proliferation and prevents budding, BMP4 expression antagonizes the effect of FGF10 (Weaver et al., 2000). Therefore BMP4-FGF10 interaction served to limit bud outgrowth during budding in development of the lung.

TGFbeta1 *Tgfbeta1* transcripts are expressed in the subepithelial mesenchyme during embryonic lung development and TGFbeta1 protein accumulates along proximal airways and in cleft formation (Lebeche et al., 1999). In the clefts, TGFbeta1 was shown to promote synthesis of ECM which is thought to prevent local branching (Heine et al., 1990). Also the addition of TGFbeta1 to lung explants in culture inhibited branching morphogenesis (Serra and Moses, 1995) and when *Tgfbeta1* is mis-expressed in the

distal lung epithelium of transgenic mice, lungs did not develop beyond late pseudoglandular stage of development (Zhou et al., 1996). Hence TGFbeta1 has been implicated as an inhibitor of cell proliferation and differentiation. An important fact about TGFbeta1 is that it has been shown to repress *Fgf10* gene expression both in a primary lung mesenchyme cells and in lung organ cultures (Lebeche et al., 1999). Therefore TGFbeta1 is another important factor that may play a vital role in modulation of FGF10 signalling in the lung, and as the regulation of *Fgf10* transcripts was studied in this thesis the TGFbeta superfamily will be discussed later.

1.2.5 FGFs in the Prostate

Member of the FGF family are key regulators of organogenesis so it is not surprising that FGFs play key roles in the development of the prostate. Both FGF7 and FGF10 play important roles in the growth and development of the prostate (Thomson and Cunha, 1999; Thomson et al., 1997), but little has been reported on the role of other FGFs during prostate organogenesis. The similarities of FGF7 and FGF10 led to the conclusion that these proteins are interchangeable. In fact *Fgf7* knockout mice have no reported change in reproductive tract development (Guo et al., 1996) suggesting that FGF10 might substitute for the lack of FGF7 protein. However in the reverse situation, *Fgf10* knockout mice do not have a prostate (Donjacour, Thomson and Cunha, manuscript in preparation) therefore FGF7 protein does not compensate for the lack of FGF10 protein. FGF7 therefore cannot replace the function of FGF10 during development of the prostate. This suggests that FGF10 is a vital factor in prostate development.

1.2.5.1 FGF7 in the Prostate

During development of the VP, *Fgf7* transcripts are expressed during periods of active growth suggesting that FGF7 is a key regulator of VP growth and development (Thomson et al., 1997). FGF7 is expressed in the mesenchyme cells and its receptor

(FGFR2 IIIb) in the epithelium of rodent prostates during development (Sugimura et al., 1996), FGF7 therefore acts in a paracrine fashion to regulate growth. FGF7 stimulates the development of VPs grown using a serum free organ culture system and FGF7 protein is mitogenic for epithelial cells (Sugimura et al., 1996). Also, addition of an anti-FGF7 antibody to a serum free organ culture system partially inhibits growth of the VP suggesting that FGF7 is required for VP growth and development (Sugimura et al., 1996). However, in *Fgf7* knockout mice no changes in the reproductive tract have been reported (Guo et al., 1996). This observation is probably due to regulatory redundancies in the processes performed by FGF7 i.e. other family members may be able to compensate in processes involving FGF7, such as FGF10. This has also been observed in *Fgf2* and *Fgf6* knockout mice as they are not only viable and fertile but are phenotypically indistinguishable from wild-type animals by gross examination (Fiore et al., 1997; Ortega et al., 1998).

1.2.5.2 FGF8 in the Prostate

FGF8 and its isoforms may play a key role in prostate carcinogenesis. Over-expression of FGF8 (Kouhara et al., 1994; MacArthur et al., 1995; Rudra-Ganguly et al., 1998) and generation of *Fgf8* transgenic mice (Daphna-Iken et al., 1998) have demonstrated the tumourigenic effect of FGF8. In a proportion of prostate cancer patients FGF8 protein (Tanaka et al., 1998) and *Fgf8* transcripts (Leung et al., 1996) may be over-expressed in the prostate. Also in prostate cancer cells *Fgf8* was regulated by testosterone at the promoter level and at the protein level (Gnanapragasam et al., 2002). It remains to be investigated whether FGF8 plays an important role in prostate development.

1.2.5.3 FGF10 in the Prostate

FGF10 plays an essential role as a paracrine regulator in the VP. Like FGF7, FGF10 is expressed in mesenchyme cells (Thomson and Cunha, 1999) and its receptor (again FGFR IIIb) in epithelial cells (Igarashi et al., 1998). During development of the VP,

Fgf10 transcripts were expressed in periods of rapid growth (Thomson and Cunha, 1999), suggesting that FGF10 is a key regulator of prostatic growth and development. The expression pattern of *Fgf10* transcripts was also shown to be more restricted than that of *Fgf7*; *Fgf10* transcripts were expressed in fewer organs compared to *Fgf7* transcripts (Bellusci et al., 1997b; Thomson and Cunha, 1999). However more recent data has identified FGF10 a key regulator in numerous processes (see table 1.1), the majority of which involve either regulation of proliferation of cells and highly folded or branched organs.

The expression pattern of *Fgf10* in the prostate (Thomson and Cunha, 1999) is similar both to the expression of *Fgf10* in the lung (Bellusci et al., 1997b) and *Bnl*, an *Fgf* homologue, in the *Drosophila* (Sutherland et al., 1996). This suggests that *Fgf10* is playing a similar role in the prostate as that observed in lung and tracheal airway development. The addition of FGF10 protein to VP organ rudiments in serum free organ culture, resulted in the growth and development of the prostate similar to that observed by the addition of testosterone alone (Thomson and Cunha, 1999). Recombinant FGF10 protein also stimulated the growth of prostatic epithelial cell lines, but did not stimulate the growth of prostatic stromal cells (Thomson and Cunha, 1999). Overall these results suggest that FGF10 functions as a paracrine regulator of prostate growth and development.

1.2.5.4 Regulation of FGF7 and FGF10 in the Prostate

FGF7 and FGF10 are expressed in the prostate during development and have been postulated as being regulators of branching morphogenesis (Thomson and Cunha, 1999; Thomson et al., 1997). Many studies have addressed their regulation in this system and as testosterone is essential for prostate development numerous studies have examined the regulation of FGF7 and FGF10 by testosterone.

Fgf7 transcripts were up regulated by androgens in prostate cells grown *in vitro* (Yan et al., 1992). However *Fgf7* transcript levels were inversely proportional to androgen concentrations *in vivo* (Nishi et al., 1996) and were repressed in response to testosterone in VPs grown *in vitro* (Thomson et al., 1997). These results concurred with studies on FGF7 protein distribution in the prostate, which showed a broad distribution of FGF7 and no significant changes in concentration as a result of androgen treatment (Nemeth et al., 1998).

It has been postulated that testosterone may regulate *Fgf10* transcript levels in the prostate (Lu et al., 1999). If testosterone increases *Fgf10* transcript levels, and thus FGF10 protein levels, it can be suggested that the addition of FGF10 protein to an organ culture system would substitute for the effect observed in VPs caused by androgens. The addition of FGF10 protein to VP organ rudiments in serum free organ culture, resulted in the growth and development of the prostate similar to that observed by the addition of testosterone alone (Thomson and Cunha, 1999). Hence these results suggest that the effect of androgens on prostate development acts via the regulation of *Fgf10* transcripts. However *Fgf10* transcript levels were not directly regulated by testosterone in VPs grown *in vitro*, and are not only inversely related to levels of circulating androgens but are also expressed in female VMPs (Thomson and Cunha, 1999). Thus it is unlikely that androgens directly regulate *Fgf10* transcript levels, however to resolve this problem the regulation of *Fgf10* transcript levels by testosterone was investigated in this thesis.

If androgens do not regulate *Fgf10* transcripts but FGF10 protein induces a similar response to androgens in organ cultures, suggesting they may act on the same pathways, how might androgens be regulating FGF10 signalling? During development of the prostate, it has been proposed that mesenchymal paracrine factors are produced constitutively and that androgens act via indirect mechanisms to control the availability or activity of these paracrine factors (Thomson, 2001). FGF10 is required for prostate development and is a mesenchymal paracrine factor. FGF10 protein may be modulated

by androgens via an indirect mechanism. In fact androgens may alter the availability of FGF10 protein by affecting FGF-heparin/HLGAG interactions. A recent study has suggested that heparin-like molecules are intimately involved in the androgen-mediated proliferation of mouse mammary tumour cells (Borgenstrom et al., 2001). Also another study has addressed a possible mechanism for androgen and heparin interaction involving FGFR signalling (Kassen et al., 2000). This study demonstrated that in the absence of DHT, FGF2 in culture was trapped in the ECM and was not available to interact with LNCaP cells. However in the presence of DHT, heparinase activity in the culture was elevated and as a result, it liberated the trapped FGF2, which stimulated the proliferation of LNCaP cells. As FGF10 has been shown to be associated with ECM (Beer et al., 1997), it is possible that an increase in heparinase caused by androgens may release the FGF10 protein and thus result in FGFR signalling.

Other factors possibly involved in modulating FGF signalling during development of the prostate are shown in Fig. 1.4. As some of these factors have been shown to regulate *Fgf10* gene expression in the lung it would be highly likely that they are involved in regulating its expression in the prostate. Numerous studies have already demonstrated that these factors are involved in prostate organogenesis (Lamm et al., 2001; Podlasek et al., 1999a; Timme et al., 1995) and in the future the mechanisms of their interactions will most probably be elucidated. However as this thesis has concentrated on the regulation of *Fgf10* gene expression by TGFbeta1, this cytokine will be discussed in more detail than the others that are briefly mentioned above. Also as the regulation of *Fgf10* gene transcription was investigated the next part of this chapter will briefly discuss gene transcription and the role that TGFbeta1 may play in regulating gene transcription.

1.3 TGFbeta in Development of the Prostate

Members of the transforming growth factor beta (TGFbeta) superfamily have been implicated in the biological processes ranging from specification of cell fate during embryogenesis to inhibition of cell proliferation during adulthood (reviewed in (Massague et al., 1992b)). In mammals there are three different *Tgfbetas*, *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* (Derynck et al., 1985; Madisen et al., 1988), which are encoded by different genes and which all function through the same receptor (Massague, 1998). TGFbetas have been implicated in playing a role in adult prostate homeostasis and ablation of androgens by castration demonstrated a role for TGFbetas during prostate regression (Kyprianou and Isaacs, 1989; Bacher et al., 1993; Itoh et al., 1998). However in the prostate it has been demonstrated that TGFbeta1 isoform is the most abundantly expressed and is also the best characterised (Timme et al., 1994). Hence the experiments performed in this thesis used recombinant TGFbeta1 protein and as a result the rest of this chapter will mainly discuss the role that TGFbeta1 plays in prostate development and disease.

1.3.1 TGFbeta1

TGFbeta1 protein is a 25 kDa homodimer composed of two disulfide linked subunits. The subunit remains associated with its N-terminal propeptide, also called latency-associated peptide (LAP), through non-covalent interactions (Munger et al., 1997). TGFbeta1 is secreted in a latent form and the mechanism by which TGFbeta1 is activated *in vivo* is not well understood. However *in vitro* culture experiments suggest that proteases play an important role in the activation of TGFbeta1 (Munger et al., 1997). In order to elicit a biological response, TGFbeta1 must be released from the latent complex so latency is one mechanism to control growth factor activity. Latency also regulates the availability of TGFbetas by limiting the diffusion from the secreting

cell thus modulating the autocrine or paracrine actions of the growth factor (Arrick et al., 1992).

In mice, targeted disruption of the *Tgfbeta1* gene results in lethal inflammation, resulting in death at approximately three weeks of age (Kulkarni et al., 1993; Shull et al., 1992). However up to about two weeks of age *Tgfbeta1* null mice appear indistinguishable from their heterozygotes and wild-type littermates. Analysis of organs revealed a marked infiltration of lymphocytes and macrophages and tissue necrosis resulting in lesions in numerous organs (Boivin et al., 1995; Kulkarni et al., 1993; Kulkarni et al., 1995; Shull et al., 1992). Many of these lesions appeared to resemble those found in autoimmune diseases, suggesting that TGFbeta1 plays a vital role in regulating immune responses in tissues. *Tgfbeta1* knockout mice have also been used to demonstrate a role for TGFbeta1 in bone (Geiser et al., 1998), hair follicle (Foitzik et al., 2000) and teeth (D'Souza et al., 1998) development and production of Langerhans cells in the skin (Borkowski et al., 1996). Recently Abate-Shen and Shen, 2000, stated that mutant mice also have defects in prostatic duct formation although there is no published data to support this (Abate-Shen and Shen, 2000).

1.3.2 TGFbeta1 Signalling

The basic signalling pathway of TGFbeta's consists of two membrane receptors and a family of receptor substrates that move into the nucleus (Fig. 1.8) (Attisano and Wrana, 2002). TGFbetas signal through a family of transmembrane serine/threonine kinases called the TGFbeta receptor family (reviewed in (Massague, 1992)). TGFbeta1 signals through an interaction of two types of TGFbeta receptor (TGFbetaR): TGFbetaRI and TGFbetaRII (Massague, 1992; Massague et al., 1992a; Massague and Chen, 2000; Wrana, 1998; Wrana et al., 1994). Both TGFbetaRI and TGFbetaRII are required for cell signalling and work together as a heteromeric complex. Initially TGFbeta1 binds to TGFbetaRII, which facilitates TGFbetaRI to recognize the ligand and TGFbetaRI is recruited into the ligand/receptor complex. Upon RI/RII/TGFbeta binding TGFbetaRI

is activated by TGFbetaRII phosphorylation, which results in downstream signalling (Fig. 1.8).

Substrates involved in the signalling of TGFbeta1 include the SMAD family of proteins (Fig. 1.8). The activated TGFbetaRI interacts with SARA (SMAD anchor for receptor)

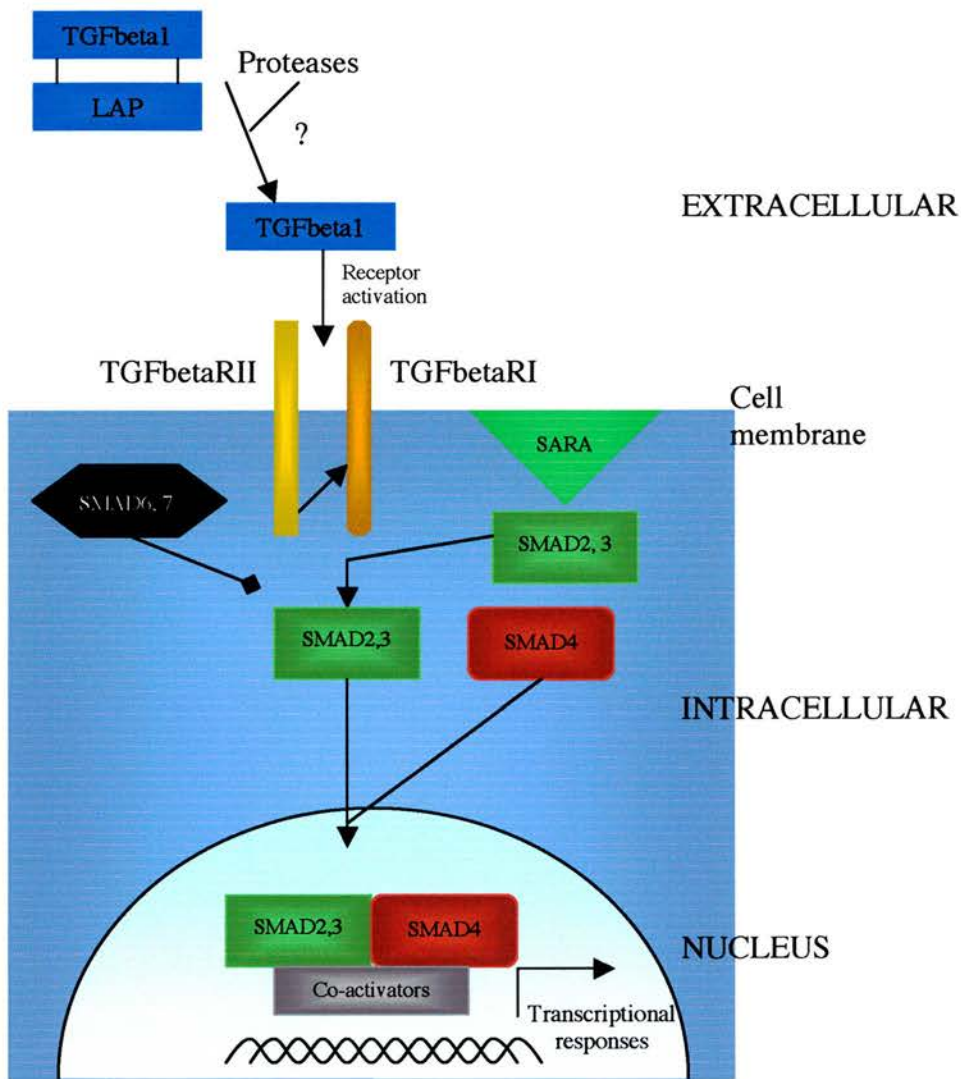


Fig. 1.8 Depicting TGFbeta1 signalling in cells as described in the text

activation) inducing the release of R-SMADs (SMAD2/-3). Phosphorylation of R-SMADs results in the accumulation of the proteins in the nucleus that aids the binding of R-SMADs to SMAD4 (a second class of SMADs, referred to as Co-SMADs) (Lagna et al., 1996; Nakao et al., 1997b). In the nucleus the R-SMAD/Co-SMAD complex associates with one of many DNA binding partners and numerous transcriptional coactivators or corepressors, thus mediating repression or activation of gene expression (Wrana, 2000; Wrana and Attisano, 1996; Wrana and Attisano, 2000). A third class of SMAD proteins, inhibitor SMADs, inhibits the effects of the other SMADs (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997a).

To summarize, in the basal state SMADs reside in the cytoplasm and upon TGFbeta1 activation of TGFbetaR SMAD2/3 are phosphorylated and form a complex with SMAD4. These complexes move into the nucleus and either alone or in conjunction with other factors, activate target genes by binding to specific promoter elements. SMADs and proteins that associate with SMADs may prove to be an important mechanism by which TGFbeta1 may regulate *Fgf10* gene expression. As this thesis has examined the regulation of the *Fgf10* transcript levels by TGFbeta1 it is important to understand the mechanisms of TGFbeta-mediated transcription. Therefore, next, an overview of factors involved in gene transcription, and the role of TGFbeta signalling in gene transcription will be discussed.

1.3.3 Gene Transcription and the Role of TGFbeta1

1.3.3.1 Gene Transcription

The regulation of gene expression is complex and not only involves the regulation of a gene at the promoter level but also involves chromatin modification, RNA polymerases, initiation cofactors, elongation cofactors and mRNA processing (reviewed by (Lee and Young, 2000)). However this thesis examines the regulation of transcription at the promoter level, hence the DNA sequences that are involved in transcriptional regulation and that are components of the transcriptional apparatus will be briefly discussed. There are at least three features common to most promoters that are involved in gene transcription: the transcription start site, the TATA box, and other regions involved in binding transcriptional regulators.

The transcription start site and the TATA box are part of the core promoter element. The average core promoter element spans from about -40 to +40 nucleotides relative to the transcription start site. This region may contain the numerous DNA sequence motifs, which in the appropriate combinations, are sufficient to promote transcription initiation by the RNA polymerase II transcriptional apparatus (Burke and Kadonaga, 1996; Emami et al., 1997; Smale et al., 1990). DNA sequence motifs within the core promoter include the TATA box, transcription factor IID (TFIID) recognition element, initiator (Inr), and the down stream promoter element (DPE) (reviewed in (Orphanides et al., 1996; Roeder, 1996; Smale, 1997; Smale, 2001)). Immediately upstream of the core promoter is a region of DNA called the proximal promoter (from about -50 to -200 bp relative to the transcription start site). The proximal promoter may contain multiple DNA binding motifs that are involved in transcription. The two most commonly found DNA binding motifs in the proximal promoter are the GC box and the CCAAT box (Jones et al., 1988; Maniatis et al., 1987). The GC box contains a region of sequence with a high GC content that is a binding site for Sp1-like transcription factor proteins (Briggs et al., 1986; Bucher, 1990; Kadonaga et al., 1987; Kriwacki et al., 1992).

Transcription factors are proteins that interacted with gene promoters and can alter transcriptional activity. Sp1 is found in all cell types of the body, and is involved in the transcription of numerous genes (Kadonaga and Tjian, 1986). Sp1 not only requires the TFIID complex for efficient stimulation of transcription *in vitro* (Pugh and Tjian, 1990; Smale et al., 1990; Tanese et al., 1991), but Sp1 can directly stabilize binding of this factor to core promoter elements (Kaufmann and Smale, 1994). Sp1 may also interact with other components of the preinitiation complex (Choy and Green, 1993). These results demonstrate that Sp1 can play an important role in gene transcription. In contrast to the GC box that mainly binds Sp1-like transcription factors, the CCAAT box binds a number of different proteins (reviewed in (Mantovani, 1998)). The activation of transcription occurs through the combined activities of numerous transcription factors acting on the core promoter. However the majority of genes are also regulated through the presence of distal binding sites (activating sequences (UAS), enhancers, and repressor sequences) to which transcriptional regulators may bind.

UAS and enhancers Transcription factors activate transcription through binding to sequences on gene promoters called UAS or enhancers (reviewed in (Blackwood and Kadonaga, 1998; Khoury and Gruss, 1983)). UAS influence transcription near to the transcription start sites while enhancers are DNA sequences that increase transcription from up to 85 kb from the transcription start site. Also enhancers work independent of their orientation.

Repressors Transcription factors repress transcription through binding DNA sequences-specific sites through various mechanisms. These include interfering with activator binding, preventing recruitment of transcriptional apparatus and modifying chromatin structure (reviewed in (Hanna-Rose and Hansen, 1996; Lee and Young, 2000))

1.3.3.2 TGFbeta-mediated Transcription

Factors that associate with SMADs may be involved in TGFbeta-mediated transcription and therefore it is important to identify such factors. An example of a protein that binds SMADs and which is a component of the first identified SMAD transcriptional complex (Huang et al., 1995) is Fast-1. Fast-1 is a member of the winged-helix family of putative transcription factors (Lai et al., 1993) and was the first component of the activin response factor (ARF) to be identified based on its ability to bind to activin response element (ARE) (Chen et al., 1996a). ARF is a DNA-binding complex that forms in response to activin or an endogenous factor, in *Xenopus* embryo explants, that binds to a 50 base pair ARE. Fast-1 resides in the nucleus (Chen et al., 1996a) and here it associates with SMAD2 and SMAD4, forming a complex that binds to ARE (Chen et al., 1997; Liu et al., 1997). Other members of the winged helix family might be DNA binding partners of SMADs.

Another factor shown to cooperate with SMADs and that may be important in context with this thesis is Sp1 (Botella et al., 2001; Brodin et al., 2000; Feng et al., 2000b; Moustakas and Kardassis, 1998). SMAD proteins have been shown to cooperate with Sp1 in the up-regulation by TGFbeta of endoglin (Botella et al., 2001), p21 (Moustakas and Kardassis, 1998), p15 (Feng et al., 2000b), SMAD7 (Brodin et al., 2000), plasminogen activator inhibitor 1 (Datta et al., 2000), and beta5 integrin (Feng et al., 2000a) gene promoters. All these genes are Sp1-dependent because the Sp1 site involved participates in transcription initiation.

An important process in TGFbeta mediated gene transcription is the binding of SMAD DNA-binding complexes with response elements within the promoter of the target genes. TGFbeta1 has been shown to cause numerous gene responses including inducing cell cycle arrest (Datto et al., 1995; Feng et al., 2000b; Hannon and Beach, 1994; Reynisdottir and Massague, 1997; Reynisdottir et al., 1995) and stimulating expression of collagen and ECM proteins (Inagaki et al., 1994; Keeton et al., 1991; Riccio et al.,

1992; Ritzenthaler et al., 1993). TGFbeta has been shown to mediate these responses possibly through the interaction of SMAD DNA-binding complexes with putative Sp1 sites located in the genes promoter. This also correlates with the fact that SMAD transcriptional complexes can contain the Sp1 transcription factor (Botella et al., 2001; Brodin et al., 2000; Feng et al., 2000b; Moustakas and Kardassis, 1998). In fact studies conducted on several different promoters have identified Sp1 sites as major TGFbeta-responsive promoter elements (Bloom et al., 1996; Botella et al., 2001; Datto et al., 1995; Greenwel et al., 1997; Li et al., 1998; Li et al., 1995; Moustakas and Kardassis, 1998). The effects observed on some of these genes also appear to require AP-1 activity (Chang and Goldberg, 1995; Chen et al., 1996b).

Interestingly, TGFbeta does not always promote gene transcription but can rapidly inhibit this process (Alexandrow and Moses, 1995; Iavarone and Massague, 1997). The rapid inhibition of gene transcription may be due to the stimulation of the TGFbeta-inducible early genes (TIEG). There are two TIEGs identified to date: TIEG1 and TIEG2. TIEG1 was identified as the product of a TGFbeta-inducible gene from osteoblastic cell population (Subramaniam et al., 1995). It is a member of the Sp1-like zinc finger transcription factor family that inhibits the growth of epithelial (Tachibana et al., 1997) and mesenchymal (Tau et al., 1998) cells. TIEG2 protein is very similar to TIEG1 and is also inducible by TGFbeta signalling and inhibits epithelial cell proliferation (Cook et al., 1998). Thus TIEGs may play an important role in the molecular machinery that regulates the growth of cells from various lineages. An important feature of TIEG1 and TIEG2 are their ability to bind to GC-rich Sp1-like *cis*-regulatory sequences (Cook et al., 1999; Cook et al., 1998; Yajima et al., 1997). This may be important owing to the fact that Sp1 is involved in promoting transcription in numerous genes (briefly described above). The amount of Sp1 protein is not affected by TGFbeta treatment (Botella et al., 2001) but TIEG transcription increases rapidly after TGFbeta treatment reaching a maximum level after about four hours (Cook et al., 1998; Subramaniam et al., 1995). The expression of TIEG transcripts decreases rapidly after reaching maximum levels. As TGFbeta inhibits gene transcription of numerous genes,

and this has been proposed to be mediated through the Sp1 site, TIEG proteins may be acting as mediators of this effect. For example, a rapid increase in TIEG may result in a rapid decrease in gene transcription of Sp1 regulated genes as TIEG may be competing for binding to the GC-rich regions normally bound by Sp1. The result of antagonizing Sp1 binding may result in a decrease in gene transcription.

Overall this data suggests that the Sp1 protein may form an active complex with TGFbeta activated proteins. The DNA-binding complexes may mediate TGFbeta-induced transcriptional changes via the Sp1 binding sites located in numerous TGFbeta regulated promoters. However TGFbeta may inhibit gene transcription through the induction of TIEG. TIEG may compete for binding of Sp1 sites thus repressing gene transcription in genes regulated by Sp1. So the overall consensus is that the TGFbeta has differing effects on different genes depending on how it influences transcription.

1.3.4 TGFbeta1 in the Prostate

TGFbeta1 was proposed as a factor involved in mesenchymal-epithelial interactions during development of the prostate (Timme et al., 1994). Numerous studies have investigated the distribution of *Tgfbeta* transcripts and TGFbeta proteins in adult tissue and during prostate organogenesis. In the adult human prostate TGFbeta1 has been localised both to the epithelial and stromal cells (Gerdes et al., 1998; Muir et al., 1994; Perry et al., 1997; Raghoe et al., 1999; Wikstrom et al., 1998), and the receptor has been localised in the epithelium, although some staining in the stroma was detected (Cardillo et al., 2000; Kim et al., 1996b; Royuela et al., 1998). This suggests that in the human adult TGFbeta1 could act as both a paracrine and an autocrine cytokine, as it may regulate processes in both stromal and epithelial cells.

In the adult rodent the expression of TGFbeta1 protein was tightly associated with the prostatic smooth muscle and RT-PCR showed that *Tgfbeta1* transcripts were also

expressed in the stroma (Nemeth et al., 1997). A preferential staining for TGFbetaR was demonstrated in epithelial cells (Kim et al., 1996a). Also during development of the rodent prostate *Tgfbeta1* transcript expression was more highly expressed in the UGM than UGE, and was predominantly immuno-localised to the UGM surrounding the developing buds throughout the perinatal period (Timme et al., 1994).

1.3.5 TGFbeta1 in Prostate Cancer

Both rat and human prostate tumours have been found to express high levels of TGFbeta1 (Thompson et al., 1992; Truong et al., 1993). It has been shown that the increase in TGFbeta1 expression is not only an early event of prostate cancer but is also associated with prostate cancer progression (Wikstrom et al., 1998). As TGFbeta1 has been shown to inhibit the proliferation rate of epithelial cells the over-expression of TGFbeta1 must be causing other effects. Hence TGFbeta1 has been implicated in cancer progression owing to its multiple effects on biological processes. TGFbeta1 has been suggested to promote tumour growth by stimulating angiogenesis, ECM modulation and by inhibiting immune responses (Arteaga et al., 1993; Chang et al., 1993; Steiner and Barrack, 1992). Hence, *in vivo*, direct effects of TGFbeta on the tumor environment, such as increased ECM, and local suppression of the immune responses may provide a growth advantage, which overrules any direct antiproliferative effects of TGFbeta1, as suggested by the effects in culture.

Research performed by Steiner and Barrack tested the direct effect of TGFbeta1 on prostate tumour cells by stably transfecting MATLyLu cells with an expression vector that codes for latent TGFbeta1, and growing them *in vitro* and *in vivo* (Steiner and Barrack, 1992). When the cells were inoculated into a host the resulting tumours were larger, less necrotic and more metastatic than inoculated MATLyLu cells that were not stably transfected with TGFbeta1. *In vitro* cell proliferation was initially inhibited by TGFbeta1 but the cells later resumed proliferation. These experiments showed that TGFbeta1 produced *in vivo* is biologically active and can promote prostate cancer

growth, viability, and aggressiveness, and that the enhanced tumor growth *in vivo* was at least in part via an effect of TGFbeta1 on the tumor cells themselves.

Also the proliferation of cancerous cells have been shown to increase in the presence of TGFbeta1. Colon carcinoma cells switch their response to TGFbeta1 with tumour progression (Hsu et al., 1994; Schroy et al., 1990). As the tumour becomes more aggressive and less differentiated the growth rate of cells is stimulated and not repressed by TGFbeta1 (Hsu et al., 1994; Schroy et al., 1990). More recently insulin-like growth factor binding protein 3 was shown to mediate the TGFbeta1-induced proliferation of highly metastatic or highly aggressive colon carcinoma cell lines (Kansra et al., 2000). TSU-Pr1 cells were originally thought to be of prostate cancer origin (Iizumi et al., 1987) and represent a cell type whose proliferation is increased in the presence of TGFbeta1 (Lamm et al., 1998). More recently TSU-Pr1 cells were shown to have probably originated from a bladder cancer cell type and not prostate cancer (van Bokhoven et al., 2001). However TSU-Pr1 cells represent a highly aggressive and relatively undifferentiated cancer cell type that is growth responsive in the presence of TGFbeta1. The fact that aggressive cancer cells are stimulated by TGFbeta1 and TGFbeta1 protein is expressed in cancer suggests that TGFbeta's not only promote cancer progressive by modulation of ECM and immunosuppressive actions but may also directly promote the growth of cancer cells.

However *Tgfbeta1* heterozygote mice, when treated with carcinogens, have an enhanced development of lung and liver cancer (Tang et al., 1998), and *Tgfbeta1* null mice also develop cancer if rescued from autoimmune disease (Engle et al., 1999). These results demonstrate that TGFbeta1 is involved in preventing cancer initiation as well as TGFbeta1's ability to promote cancer progression, described above.

1.3.6 Function of TGFbeta1 in the Prostate

The function of TGFbeta1 in the lung was described above and TGFbeta1 may play similar roles in the prostate. *In vitro*, TGFbeta1 inhibits the proliferation and induces cell death of both normal prostatic epithelial and stromal cells derived from rodents and humans (Ilio et al., 1995; Kassen et al., 1996; Story et al., 1993; Sutkowski et al., 1992). TGFbeta1 has been shown to inhibit prostate epithelial cells in G1 by stimulating the production of cyclin-dependent inhibitors (Robson et al., 1999), but at low concentrations TGFbeta1 causes small increases in cell proliferation in prostate cells (Collins et al., 1996; Robson et al., 1999). TGFbeta1 has numerous other effects on prostate stromal cells; it induces the expression of SM (Peehl and Sellers, 1997) and has been shown to inhibit AR action in a prostate smooth muscle cell line (PS-1) by inducing a nuclear to cytoplasmic distribution of AR (Gerdes et al., 1998). Also TGFbeta downstream signalling molecule, SMAD3, has been shown to directly bind to AR and may either promote (Kang et al., 2001) or inhibit (Hayes et al., 2001) AR signalling in prostate cell lines. Also the binding of SMAD3 to AR may inhibit TGFbeta signalling (Chipuk et al., 2002). Hence several possible roles for TGFbeta1 in affecting prostate development and homeostasis in adulthood have been demonstrated in cells cultured *in vitro*.

Early studies of rodent VPs in castration-induced androgen withdrawal demonstrated a role for TGFbeta1 during prostate regression (Bacher et al., 1993; Itoh et al., 1998a; Kyprianou and Isaacs, 1989). It was shown that there was a temporal correlation between the expression of TGFbeta transcripts and the activation of programmed cell death in the rat ventral prostate after castration (Kyprianou and Isaacs, 1989). TGFbeta transcript levels were elevated after castration and androgen replacement suppressed these levels back to the low level observed in the intact control (Kyprianou and Isaacs, 1989). To test if TGFbeta activates programmed cell death observed in the rat prostate after castration TGFbeta protein was administered directly to the VP. *In vivo* treatment with TGFbeta lead to a significant decrease in DNA content suggesting it was able to

induce cell death of at least a portion of epithelial cells. However the same effect is not observed in isolated prostatic epithelial or stromal cells (Itoh et al., 1998a) suggesting stromal-epithelial interactions were required for the effect observed in the prostate. This, along with evidence in prostate developmental models, provides a hypothesis that a network of locally produced growth factors that are potentially regulated by androgens is required for prostate growth and differentiation.

TGFbeta1 has also been shown to repress growth and branching morphogenesis of numerous organs including the prostate and seminal vesicles (Itoh et al., 1998a; Tanji et al., 1994). TGFbeta1 had no or little effect on branching morphogenesis in the absence of testosterone but it inhibited testosterone-induced branching morphogenesis in both the seminal vesicle and VP. These data suggests that TGFbeta1 has an integral role in regulating growth and development of the VP. As a result TGFbeta1 will be examined not only in the regulation of *Fgf10* transcript levels but also as a factor that regulates growth and development of the VP.

1.4 Summary

The development of the prostate is controlled by mesenchymal-epithelial signaling and is dependent on the presence of androgens (Chung et al., 1991; Cunha et al., 1983; Tenniswood, 1986). Numerous molecules are involved in mesenchymal-epithelial signaling and have been shown to regulate prostatic organogenesis (Itoh et al., 1998a; Lamm et al., 2001; Podlasek et al., 1999a; Thomson and Cunha, 1999). Two factors shown to have opposing roles in regulation of prostate growth are FGF10 and TGFbeta1 and both have been postulated as being regulated by androgens (Bacher et al., 1993; Itoh et al., 1998a; Kyprianou and Isaacs, 1989; Lu et al., 1999).

FGF10 is a mesenchymal-derived paracrine factor that may regulate the pattern of epithelial growth during development of the numerous organs including the prostate, lungs and mammary glands. In the lung FGF10 acts as a chemotactic agent for epithelial growth (Park et al., 1998), and *Fgf10* is expressed in mesenchymal cells surrounding branching epithelial tips, suggesting *Fgf10* is strictly regulated and is involved in patterning of the epithelium architecture (Thomson and Cunha, 1999; Bellusci et al., 1997b). In fact, *Fgf10* is regulated by factors produced in the epithelium and in cells surrounding the epithelium (Bellusci et al., 1997b). A small number of studies have investigated the regulation of *Fgf10* in the prostate. In prostate cells *Fgf10* transcript levels have been shown to be up-regulated by testosterone (Lu et al., 1999), however no direct testosterone-mediated regulation of *Fgf10* transcript levels in organ rudiments grown *in vitro* was demonstrated (Thomson and Cunha, 1999). Factors shown to regulate *Fgf10* in the lung need to be investigated in the prostate to understand prostate organogenesis and to establish whether mechanisms of branching morphogenesis are maintained throughout branched organs. One such factor is TGFbeta1.

TGFbeta1 is expressed in prostatic mesenchymal cells directly surrounding the tips of prostatic epithelial buds (Timme et al., 1995). TGFbeta1 is expressed during development of the prostate (Itoh et al., 1998a; Timme et al., 1994) and during prostate cancer (Thompson et al., 1992; Truong et al., 1993). TGFbeta1 has been postulated as regulating prostatic growth by inducing cell death (Martikainen et al., 1990) and/or inhibiting cell proliferation (McKeehan and Adams, 1988). More recently TGFbeta1 has been shown to repress *in vitro* VP growth by inhibiting branching morphogenesis (Itoh et al., 1998a). Furthermore, evidence has suggested that TGFbeta1 represses *Fgf10* transcript levels in numerous cell types and in lung explants (Beer et al., 1997; Lebeche et al., 1999). Hence TGFbeta1 may in part inhibit growth of VPs by repressing *Fgf10* gene expression. Therefore TGFbeta1 may play an opposing role to FGF10 during the development of the prostate, and the regulation of *Fgf10* by TGFbeta1 may be one mechanism of branching morphogenesis.

Hence, the objective of this thesis is to provide further insight into the molecular mechanisms underlying prostate development. More specifically, this thesis aims to 1) investigate whether *Fgf10* is regulated by TGFbeta1 in prostate cells and organs, 2) characterise the *Fgf10* promoter to identify regions that regulate *Fgf10* gene expression, and to determine whether any regions of the promoter mediate the effects of TGFbeta1 and 3) determine the role of TGFbeta1 in prostatic growth during development. Overall, we aim to have provided an insight into the regulation of *Fgf10* and hence to have identified a possible mechanism of branching morphogenesis in the prostate.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Agarose gel electrophoresis was performed using Bio-Rad equipment.

Automated sequencing gels were run using an ABI 310 Genetic Analyser by the Human Reproductive Sciences Sequencing Service, MRC, UK.

Autoradiography was performed using Kodak Biomax MS X-ray film, with Amersham Biosciences Hypercassette intensifying screens.

Colourmetric assays were read on Labsystems original Multiscan EX.

Confocal microscopy was performed on a Zeiss LSM 510 Laser scanning microscope and lower power (10X) fluorescent microscopy was performed using the Provis Olympus AX70 with a Kodak DS330 digital camera.

Glass bottom microwell dishes (Cat No. P35G-0-10-C) were purchased from Plastek® Cultureware, MatTek Corporation, USA.

Luciferase assays were performed on a EG& G Berthold microplate luminometer.

Nucleic acid concentrations were determined using a WPA lightwave spectrophotometer.

Paraffin embedding was performed on a Leica TP 1050, the tissue embedding center was purchase from Reichert-Jung and tissue sections were cut using the Leica Microtome RM 2135.

PCR was performed using a Peltier Thermal Cycler PTC 200 from MJ Research.

Phospho Screen and Phosphoimager Storm 860 were purchased from Molecular Dynamics.

Platform Shaker STR6 was purchased from Stuart Scientific.

RNase protection sequencing gels (CastAway® Precast Sequencing Gel, 6 % polyacrylamide 1 X TBE 7 M Urea (Cat NO. 401090)) were run and dried using Stratagene CastAway® Sequencing Device and CastAway® Gel Dryer.

Western analysis was performed using the Invitrogen™, NuPAGE™ Electrophoresis System.

2.1.2 Computer Software

Sequence analysis and alignments were performed using GeneJockey II Sequence Processor (P. Taylor, BIOSOFT) and VISTA analysis (<http://www-gsd.lbl.gov/vista/>). Sequence similarity searches were performed using NIX from the UK Human Genome Mapping Project Resource Centre (<http://www.hgmp.mrc.ac.uk/>), and BLAST from NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phospho-images were quantitated using image Quant Version 1.2 (Molecular Dynamics). Images were captured on a Macintosh G3/G4™ and © Apple Computer, Inc. and manipulated with Adobe® Photoshop® 5.0 PowerPC™.

2.1.3 Enzymes

T7 and T3 RNA polymerases, T4 polynucleotide kinase, bovine alkaline phosphatase, T4 DNA ligase, *Taq* DNA polymerase, *RNase*-free *DNase* I and restriction endonucleases were purchased from Promega and used with the supplied buffers.

SUPERSCRIPT™ II *RNase H* Reverse Transcriptase was purchased from Invitrogen™, Life Technologies and used with the supplied buffers.

DNase-free *RNase* cocktail was purchased from Ambion, Inc.

Proteinase K was purchased from Sigma-Aldrich, Inc.

2.1.4 Nucleic Acids

Generuler™ 1kb DNA ladder and Φ X174 DNA/BsuRI (*Hae*III) marker were purchased from MBI Fermentas.

Ultrapure NTP Set, Nucleoside 5'-Triphosphate (NTPs), was purchased from Amersham Biosciences, Inc.

2'-deoxynucleoside-5'-triphosphates (dNTPs) were purchased from Promega Inc.

Radioisotope redivue uridine 5'-[α -³²P]-triphosphate, triethylammonium salt was purchased from Amersham Biosciences, Inc.

Synthetic oligonucleotides were synthesised by either Sigma-Genosys Ltd. or MWG-Biotech AG.

2.1.5 Chemicals

5'-bromo-4'-chloro-3'-indolyl- β -D-galactopyranoside (X-gal) was purchased from CALBIOCHEM®.

Recombinant RNase Inhibitor (Cat. No. N251A) was purchased from Promega.

All other chemicals for standard solutions were obtained from BDH, Sigma-Aldrich or Invitrogen™, life technologies and were of analytical grade.

2.1.6 Bacterial Strain Genotypes

DH5 α : endA1, hsdR17, (rk⁻mk⁻), supE44, thi-1, recA1, gyr A, (Nal^r), relA1, Δ (lacIZY A-argF)U169, deoR, (ϕ 80dlac Δ (lacZ)M15), competent cells purchased from Invitrogen™, Life Technologies (Cat No. 18258-012)

XL-1 blue recA1, endA1, gyrA96, thi-1, hsdR17, supE44, reiA1, lac[F'proABIlac19Z Δ M15Tn10(Tet^r)], competent cells purchased from Stratagene (Cat No. 200130)

2.1.7 Bacterial Culture Media

Luria Agar (LA) Luria broth plus 1.5 % (w/v) agar

Luria Broth (LB) 10 g l⁻¹ bacto-tryptone, 5 g l⁻¹ bacto-yeast extract, 5 g l⁻¹ NaCl, adjusted to pH 7.0 with 5 N NaOH

SOC media 2 % bacto-tryptone, 0.5 % bacto-yeast extract, 20 mM glucose, 2.5 mM KCl, 10 mM MgCl₂,

2.1.8 Mammalian Cell Lines

- NIH-3T3 Clonally derived cell line from mouse embryo fibroblasts (Muller et al., 1984)
- PS-1 Clonally derived from prostate stromal cells in the VP (Gerdes et al., 1996)

2.1.9 Organ and Cell Culture Media and Equipment

All flasks, cryotubes and pipettes were purchased from Falcon®, NUNC™, or Corning Inc. (COSTAR®).

Controlled Process Serum Replacement – type 1 (CPSR, Cat No. C8905) was purchased from Sigma-Aldrich, Ltd.

Dulbecco's Modern Eagle media (DMEM) with glutamax I (Cat No. 31966-021), DMEM/NUT.MIX F-12 with glutamax II (Cat No. 4133-026), Penicillin-Streptomycin (Cat No. 15240-062), Trypsin-EDTA (Cat No. 25300-062), Foetal Bovine Serum (Cat No. 10106-169), Bovine Transferrin (Cat No. 11107-018), and Crystalline Bovine Insulin (Cat No. 13007-018) were purchased from Invitrogen™, Life Technologies.

GenePORTER™ Transfection Reagent (Cat No. T201075) was purchased from Gene Therapy Systems.

Manipulations were carried out in statutory Class II tissue culture hoods.

Millicell® CM Culture plate inserts (Cat No. PICM03050) were purchased from Millipore.

2.1.10 Immunohistochemical and Western Reagents

Hybond-P Protein Transfer Membrane (Cat No. RPN303F) and ECF™ substrate (Cat No. 1067873) were supplied by Amersham Biosciences.

Normal Goat and Rabbit serum were supplied by Diagnostic Scotland.

NuPAGE™ 10 % Bis-Tris Gel 1.0 mm X 15 well (Cat No. 1091713), Loading Dye (Cat No. 46-5030), Sample Antioxidant (Cat No. 46-5027), 20 X MOPS SDS Running Buffer (Cat No. 46-5025) and 20 X Transfer buffer (Cat No. 46-5033) were supplied by Invitrogen™, Life Technologies.

Pre-stained SDS Page standards (broad-range) (Cat No. 161-0318) were purchased from Bio-Rad.

Table 2.1 Antibodies used and their dilutions in Westerns and Immunohistochemistry

Antibody	Source	Species	Epitope	Dilutions	
				Westerns	Immuno-histochemistry
Anti-β tubulin	Sigma-Aldrich, Inc (Cat No. T5293)	mouse	monoclonal		1:300
anti-cytokeratin, pan (mixture) mouse	Sigma-Aldrich, Inc (Cat No. C2562)	mouse	monoclonal		1:300
Anti-Actin, α Smooth Muscle	Sigma-Aldrich, Inc (Cat No.	mouse	monoclonal	1:1000	1:5000

	Inc (Cat No. A2547)				
Anti-androgen receptor	Santa Cruz supplied by Autogen Bioclear (Cat No. sc-816)	rabbit	polyclonal	1:300	1:400
Anti-BrdU	Fitzgerald Industries International, Inc (Cat No. 20-BS17)	sheep	polyclonal		1:200
Anti-mouse CY5	Sigma-Aldrich, Inc	goat			1:60
Anti-rabbit FITC	Sigma-Aldrich, Inc	goat			1:200
Anti-rabbit IgG (whole molecule) Alkaline Phosphatase (AP)-conjugate	Sigma-Aldrich, Inc (Cat No. A-3687)	goat		1:20000	
Anti-mouse IgG (Fab specific) Alkaline Phosphatase (AP)-conjugate	Sigma-Aldrich, Inc (Cat No. A-1293)	goat		1:20000	
Anti-rabbit biotinylated	Vector Laboratories	goat			1:500
Anti-sheep biotinylated	Vector Laboratories	rabbit			1:500
Streptavidin-Alexafluor 488	Molecular Probes				1:200

2.1.11 Kits

ECF substrate kits were purchased from Ambion, Inc.

GeneClean® II Kits (Cat No. 1001-400) were purchased from Anachem, Ltd.

Luciferase Assay System Kits (Cat No. E1801) were purchased from Promega.

Mini Quick Spin RNA Columns (Cat No. 814 427) were purchased from Roche Diagnostic Corp.

pGEM®-T Easy Vector System I Kit (Cat No. A1360) was purchased from Promega.

Qiaprep® Spin Miniprep Kits (Cat No. 27140) were purchased from Qiagen®.

Qiagen Maxiprep Kits (Cat No. 12162) were purchased from Qiagen®.

Advantage™ 2 PCR Kits (Cat No. K1910-y) were purchased from BD Biosciences Clontech.

2.1.12 Standard Solutions (A to Z)

β-Galactosidase Enzyme Assay	200 mM sodium phosphate buffer, pH 7.3,
2X Buffer	2 mM magnesium chloride,
	100 mM β-mercaptoethanol,
	1.33 mg ml ⁻¹ o-nitrophenyl β-D-galactopyranoside
	(ONPG)

Boiuns fixative	75 ml saturated picric acid,
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	25 ml 40 % (v/v) Formaldehyde, 5 ml glacial acetic acid
Hybridisation Buffer (5X) (for RNase protection assay)	200 mM PIPES pH6.4, 2 M sodium chloride, 5 mM EDTA
Paraformaldehyde fixative	4 % (w/v) paraformaldehyde in PBS
PBS Sigma®	phosphate buffered saline tablet supplied by
Protein Extraction Solution for cells	20 mM HEPES pH 7.9, 150 mM sodium chloride, 1 mM EDTA, 1 % (v/v) NP40, 0.5 mM DTT, 1 mM PMSF, 20 $\mu\text{g ml}^{-1}$ aprotinin, 10 $\mu\text{g ml}^{-1}$ leupeptin, 20 $\mu\text{g ml}^{-1}$ pepstatin
Protein Extraction Solution for tissue	10 mM HEPES pH 7.9, 10 mM KCl,, 0.1mM EGTA, 0.1mM EDTA, 1mM DTT, 0.5 mM PMSF, 20 $\mu\text{g ml}^{-1}$ aprotinin, 10 $\mu\text{g ml}^{-1}$ leupeptin,

	20 $\mu\text{g ml}^{-1}$ pepstatin
RNase Protection Digestion Buffer	300 mM sodium chloride, 10 mM Tris pH 7.5, 5 mM EDTA
RNase Protection Loading Buffer	80 % (v/v) formamide, 10 mM Tris pH 7.5, 5 mM EDTA. Xylene Cyanole FF (few grains)
Sequencing Gel Fixative	5 % (v/v) methanol, 5 % (v/v) glacial acetic acid, in dH ₂ O
Solution D (50 ml) (Chomczynski and Sacchi, 1987)	25 g guanidinium thiocyanate, 29.3 ml dH ₂ O, 1.76 ml 0.75 M sodium citrate pH 7.0, 2.64 ml 10% (v/v) sarkosyl NL 30, 38 μl β -mercaptoethanol
SSC (20 X)	3 M NaCl, 0.3 M sodium citrate pH 7.0
TAE (50 X)	2 M Tris acetate, 50 mM EDTA pH 8.0
TBST	0.15 M sodium chloride, 0.1% Polyoxyethylenesorbitan Monolaurate (Tween20), in Tris pH 7.4

2.2 Methods

2.2.1 Mammalian Cell Culture

2.2.1.1 Isolation and Culture of Primary Mesenchyme Cells from the Female Urogenital Sinus

Cells used for primary culture were derived from micro-dissected urethral and VMP stroma from P0 Wistar rat female urogenital sinus (UGS). Between 13 and 18 UGSs were micro-dissected into VMP and urethral stromal tissue. VMPs and urethral stroma tissue were put into separate 1.5 ml eppendorf with 1 ml serum free DMEM with glutamax I supplemented with 1 X penicillin/streptomycin (penicillin G sodium (100 U ml^{-1}), streptomycin sulphate ($100 \mu\text{g ml}^{-1}$), and amphotericin B ($0.25 \mu\text{g ml}^{-1}$)) and 1 mg ml^{-1} collagenase. Incubating and shaking vigorously, at 37°C for 45 minutes dissociated the stromal cells. Dissociated VMP (VMPC) and urethral stromal (URSC) cells were cultured in T75 flasks in the presence of 10 ml DMEM with glutamax I supplemented with 10 % FBS (foetal bovine serum) and 1 X penicillin/streptomycin. The cell media was replaced with fresh DMEM (10 % FBS) every two days until they were passaged at 95 % confluence. VMPC were passaged a maximum of three times and URSC were passaged a maximum of 4 times.

At approximately 95 % confluence, cells were washed twice in PBS and detached from the flask by incubation with 2.5 ml of 1 X trypsin-EDTA for 5 minutes at 37°C . Trypsin-EDTA was neutralised by the addition of fresh media containing 10 % FBS and

cells were collected by centrifugation at 1000 rpm. Cells were re-suspended in 10 ml of fresh media and were seeded at a ratio of 1:10 into new flasks (grown at 37 °C in a 5 % CO₂ incubator).

Cells were treated with 10⁻⁸M testosterone and/or TGFbeta1 (5 ng ml⁻¹) for varying times to study the regulation of endogenous *Fgf10* transcript levels. They were also used in transfection studies to identify elements that regulate the *Fgf10* promoter and to test the response of the promoter constructs to TGFbeta1.

2.2.1.2 Culture of Cell Lines

Cell lines were grown in DMEM with glutamax I supplemented with 10 % FBS (foetal bovine serum) and 1 X penicillin/streptomycin. Cells were passaged in the same way as for primary cells a maximum of 15 times. NIH-3T3 and PS-1 cells were treated with 10⁻⁸ testosterone and/or TGFbeta1 (5 ng ml⁻¹) for varying times to study the regulation of endogenous *Fgf10* transcript levels. NIH-3T3 cells were also used in transfection studies to identify elements that regulate the *Fgf10* promoter.

2.2.1.3 Transfection Studies

Transfection

Cells were passaged, as above, and cultured overnight in 6 well dishes, at a concentration of 1 X 10⁵ cells per well at 37 °C in a 5 % CO₂ incubator. 0.1 µg of plasmid DNA (luciferase expressing vector with promoter element) mixed with 0.02 µg of a pSV-β-galactosidase control vector (Promega) in 10 µl of deionised H₂O, was incubated with 8 µl GenePORTER™ and 10 µl PBS, for 15 minutes at room temperature. Cells were rinsed twice in serum free cell culture media (DMEM with glutamax I and 1 X penicillin/streptomycin) and finally covered with 800 µl of serum

free media. After 15 minutes 200 μ l of serum free media was mixed with the plasmid/GenePORTER mixture and pipetted into each well. The cells were incubated for 6 hrs at 37 °C in 5 % CO₂, then washed twice with serum-containing media (DMEM with glutamax I, 10 % CPSR, and 1 X penicillin/streptomycin). Cells were incubated for 36 hrs in 2 ml of serum containing media, then rinsed twice in PBS and harvested by scraping in the presence of 1 X Reporter Lysis Buffer. Luciferase activity was measured and normalised to β -galactosidase activity.

Luciferase Assay

20 μ l of cell extract in 1 X Reporter Lysis Buffer was used per reaction. As a negative control, the same dilution of the cell extract was made from cells that have been transfected with a luciferase-expressing vector with no promoter elements. Luciferase activity was measured on the EG&G Berthold Microplate Luminometer, as per manufacturers' instructions, and repeated twice per cell extract.

β -galactosidase Assay

50 μ l of cell extract in 1 X Reporter Lysis Buffer was used per reaction. As a negative control, the same dilution of the cell extract was made from cells that have not been transfected with β -galactosidase gene. 50 μ l of the cell extract was mixed with 50 μ l of β -galactosidase enzyme assay buffer (2 X) in 96 well plates. The plate was incubated at 37 °C until a faint yellow colour had developed. The reaction was stopped by adding 150 μ l of 1 M sodium carbonate. The absorbance was read using the Labsystems Original Multiscan EX at 402 nm. Each reaction was performed in duplicate.

2.2.2 *In Vitro* Organ Culture

Female UGTs and male VPs were micro-dissected from P0 Wistar rats and grown in serum-free organ culture (DMEM/NUT.MIX F-12 supplemented with transferrin (20 $\mu\text{g ml}^{-1}$), insulin (20 $\mu\text{g ml}^{-1}$), and 1 X penicillin/streptomycin). Female UGTs and VPs were positioned in a drop of media on Millicell® CM filters that were floated on 1 ml of culture media in 4 Well NUNCLON™ plates. Organ rudiments were cultured at 37 °C in 5 % CO₂ for up to six days. Regulation of growth and proliferation of epithelial and stromal cells in VPs were examined by incorporation of BrdU (0.01 mg ml⁻¹) (incubated for 2 hours at 37 °C) on day three or six of culture. VPs were imaged under light-field illumination prior to fixation, and immunohistochemistry was used to visualise BrdU incorporation. To study regulation of endogenous *Fgf10* transcript levels, organ rudiments were cultured in the absence and presence of testosterone (10⁻⁸ M) and/or TGFβ1 (10 ng ml⁻¹) for either seven hours, three days or six days. Organ rudiments were imaged under light-field illumination at the end of culture prior RNA extraction.

2.2.3 Total RNA Extraction

Total RNA was extracted from mammalian cell types or tissue using a modified AGPC (acid guanidinium thiocyanate-phenol-chloroform) method (Chomczynski and Sacchi, 1987). The protocol was scaled up so that 500 μl of solution D was used per 75 cm² flask, or approximately 20 cultured VPs. The following protocol was used for harvesting one 75 cm² flask of cultured primary VMP cells.

The cells were washed three times in PBS then lysed with 500 μl of solution D. The flask was incubated at RT for 10 minutes and scraped using a cell scraper. The cell lysate was removed and put into a 2.0 ml eppendorf. To this was added 50 μl 2 M sodium acetate pH 4.0, 500 μl of unbuffered phenol, and 100 μl chloroform: iso-amyl alcohol (49:1). The sample was mixed after each chemical was added, then chilled on

ice for 15 minutes, and centrifuged at 15 000 rpm for 15 minutes at 4 °C. The aqueous top phase was pipetted into a clean tube, and 1.2 ml of 100 % ethanol was added. The sample was incubated at -70 °C for approximately 30 minutes, and spun at 15 000 rpm for 15 minutes at 4 °C. The RNA pellet was washed in 70 % ethanol, spun at 15 000 rpm for 5 minutes, air-dried for 15 minutes, and resuspended in 200 µl of ddH₂O. The concentration of RNA was determined by UV spectrophotometry at 260nm. Integrity of the RNA was checked by gel electrophoresis; 1 µg RNA (in 20 µl of ddH₂O) was heated to 70 °C for 10 minutes, cooled on ice, spun down, and loaded onto a 0.6 % (w/v) agarose gel. The gel was stained with ethidium bromide and photographed. The presence of 28S and 18S ribosomal RNA bands confirmed RNA integrity

2.2.4 cDNA Synthesis

1 µg of total RNA was reverse transcribed in the presence or absence of reverse transcriptase (SUPERScript™ II RNase H⁻ Reverse Transcriptase; Invitrogen™, life technologies), as per manufacturers' instructions. The completed reactions were diluted with 80 µl of ddH₂O. The quality of cDNA was always checked using PCR for HPRT (HPRT-L-CCTGCTGGATTACATTAAAGCACTG, HPRT-R-CTGAAGTACTCAT TATAGTCAAGGG).

2.2.5 Polymerase Chain Reaction

In a 20 µl reaction volume, 5 ng of plasmid DNA or 7.5 µl of cDNA was mixed with 1 X PCR buffer, 15 mM MgCl₂, 0.2 mM dNTPs, 10 pmoles of each primer, and 0.5 U *Taq* DNA polymerase. Typically 35 cycles of PCR were performed using the following conditions: samples preheated to 94 °C for 1 minute, then a standard 35 cycles of 94°C for 30 seconds, T_m for 30 seconds and 72 °C for 1 minute per kb to be amplified. A final extension at 72 °C for 15 minutes was performed and samples were incubated at 15 °C. T_m refers to the annealing temperature in °C used for the oligonucleotides based upon

the formula $T_m = 4 \text{ (No. of G/C bases in the oligonucleotide)} + 2 \text{ (No. of A/T bases in the oligonucleotide)}$. PCR products were visualised by agarose gel electrophoresis and ethidium bromide staining.

Table 2.2 Primers for RT-PCR and cloning riboprobes

Probe/construct	Primer (5' TO 3')	
	Forward	Reverse
TGFbeta1	CGTGCTAATGGTGGACCGCAACAAC	AAGACAGCCACTCAGGCGTATCAGT
TGFbeta2	CTGCTGTACCTTCATACCGTCTA	CAATAGGCGGCATCCAAA
TGFbeta3	AGTGGCTGTTGCGGAGAGAGTCC	GCACACAGCAGTTCTCCTCC
TGFbeta receptor type I	CTGCCTGCTTCTCATCGTGTTG	ATGATAATCCGACACCAACCACAGC
Probe3	CTACAGTGGAAAACAAGTGAA	GCGGCTGGCGTTTTTGTGTTGGG
Probe4	TTCCCAGCAGCTTAGGGTTTCA	ACATCTGCGCCCCTCTGCGGTT
Probe5	TTCCCAGCAGCTTAGGGTTTCA	GTGTCTCTTTGGAGTTGTCAGAAC
Probe6	GCCCCCAAACAAGAAGT	GTCTCTCCAGTCTCAGGGTGAA

2.2.6 Cloning of DNA

2.2.6.1 Subcloning of Restriction Fragments

Preparation of Insert DNA.

DNA was digested with the required restriction enzymes and subjected to agarose gel electrophoresis to resolve the correct restriction fragment. The desired DNA fragment was extracted from the gel using the GeneClean II kit, as per the manufacturers' instructions. The extracted DNA was resuspended in 10 µl of ddH₂O and its' concentration estimated by running a 1 µl aliquot on an agarose gel against a known amount of DNA ladder.

Preparation of Plasmid DNA

Plasmid DNA was digested with the required restriction enzymes and subjected to agarose gel electrophoresis to check for complete digestion.

If the restriction digestion produced self-cohesive ends the following procedure was used. The restriction enzyme was heat inactivated at 65 °C for 10 minutes, the vector DNA was purified by extraction with phenol:chloroform:IAA (25:24:1) and precipitated with 1/10 volume sodium acetate pH 5.2 and 2.5 volumes of ethanol. The vector DNA was resuspended in an appropriate volume of ddH₂O and then de-phosphorylated using bovine alkaline phosphatase, as per the manufacturers' instructions. The vector was then purified by agarose gel electrophoresis and gel extracted using the GeneClean II kit. The digested vector was resuspended in ddH₂O to a concentration of approximately 50 ng μl^{-1} .

Vectors prepared without self-cohesive ends were purified by agarose gel electrophoresis and gel extracted using the GeneClean II kit. The volume of the vector DNA solution was adjusted to a concentration of approximately 50 ng μl^{-1} .

Ligation

Ligation of the restriction fragments into the prepared vectors was performed using appropriate insert: vector ratios (as suggested by manufacturers' instructions), 1 X T4 DNA ligase buffer, and 0.05 U μl^{-1} T4 DNA ligase in a 10 μl volume. Blunt-end ligation was performed at 4 °C overnight and sticky-end ligation was performed at room temperature for four hours or at 16 °C overnight. 2 μl of the ligation mix were transformed into 50 μl of DH5 α (Gibco, UK) or XL-1 blue (Statagene, UK) *E.coli*, as per the manufacturer's instructions.

Cloning of PCR Products

PCR products were purified from agarose gels using the GeneClean II kit, and cloned into a T-tailed vector using the Promega pGEM®-T Easy Vector, as per manufactures instructions, before transformation into DH5 α or XL-1 blue *E. coli*.

2.2.6.2 Cloning of *Fgf10* Promoter Constructs

Preparation of pA3Lucm

pA3Lucm was derived from pA3Luc, a vector designed to prevent cryptic plasmid transcription (Wood, 1989). The *BglIII* restriction site of pA3Luc was cut by restriction digestion, the protruding ends were filled in and blunt ended with T4 DNA polymerase (as per manufacturers instructions, Promega), and ligated (section 2.2.6.1). The vector was restricted digested with *KpnI* and *HindIII* and ligated with a polylinker, containing *KpnI*, *SacI*, *MluI*, *NheI*, *SmaI*, *XhoI*, *BglIII* and *HindIII*, digested from Full pGL3 (supplied by Justin Grindley). 2 μ l of ligation reaction mix was transformed into XL-1 blue cells, as per manufacturers' instructions, sequencing checked the insert was correct, then the plasmid was grown and extracted using a Qiagen® plasmid maxi kit (section 2.2.7.2). pA3Lucm was used to make all the constructs described below.

Preparation of 5' Deletion Constructs

Full length and 5' deleted sequences from the *Fgf10* promoter were amplified by PCR using the sequence specific primers detailed in table 2.3. Details of the constructs can be found in table 2.4. Using the Advantage™ 2 PCR kit, samples were preheated to 94 °C for 1 minute, then 5 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds and 69 °C for 1 minute. A final extension at 72 °C for 15 minutes was performed and samples were incubated at 15 °C. PCR products were ligated into pGEM T-easy vector system I, as per manufactures instructions. Inserts were released by restriction digestion using *SpeI*

and *Bam*HI and ligated, as described in section 2.2.6.1.4., into pA3Lucm which had been digested with *Nhe*I and *Bgl*III. 2 µl of ligation reaction mix was transformed into XL-1 blue cells, as per manufacturers' instructions, sequencing checked the insert was correct, then the plasmid was grown and extracted using a Qiagen® plasmid maxi kit (section 2.2.7.2.).

Table 2.3 *Fgf10* sequence specific primers

Primer name	Position on <i>Fgf10</i>	Primer Sequence
FullR	+600,+622	AACTCTCGGCACTGGAAATTGTC
FullF	-5853,-5834	ATTCCCATCCCTAATGTCA
ΔA F	-5221,-5201	TCCTTTCTACCTTGGCCACA
ΔA-C F	-3136,-3116	CAAATCGTCGAATTTAACAG
ΔA-D F	-2447,-2427	TACCTCACTATAGGTAGAAC
ΔA-F F	-307,-287	GGACGCCGGAGCCGCAATTA
ΔA-G F	-63,-43	AATGAGGTTTGACCAGCAGA
ΔA-ΔA5'UTRF	-63,-41	GAAGATCTTCTGAGGTTTGACCAGCAGAGG
ΔA-ΔA5'UTRR	+50,+72	CCCAAGCTTGGGGCGGCTGGCGTTTTG
SS F	-307,-289	GAAGATCTGGACGCCGGAGCCGCAATTA
SS R	+482,+502	CCCAAGCTTCGCTCCGTCCTCGGATC
FULLSPE F	-5853,-5834	CACTAGTATTCCCATCCCTAAT GTCA
ΔF R	-2103,-2123	CGGATCCAATTCAAATGTTTGAATTGACAACC
ΔF-C R	-3573,-3593	CGGATCCATAGATACAATCAACCACGTTG
ΔF-B R	-4712,-4732	CGGATCCAAGCAGATTCTTTCAAATGATATGAA
ΔF-A R	-5473,-5493	CGGATCCGTGATATTTGTAATATTTTGTTAGA
+SP1 F	-214,-197	CACTAGTAAGGCATCGCGCACTCC
-SP1 F	-171,-158	CACTAGTTCCCCGGTTCCTTC
ΔA-ΔΔSP1bglIIIF	-171,-152	GAAGATCTTCTCCCCGGTGCCCTCCCCCTC
ΔA-ΔΔSP1hindIIIR	+482,+502	TTCAAGCTTACTCTCAGGGTGAACAGTGGACG
ΔA-ΔΔSP1speF	-2447,-2425	GGACTAGTACCTCACTATAG GTAGAAC
ΔA-ΔΔSP1bglIR	-203,-183	GGAAGATCTTCCGATATTTGGGCGAGAGGAGTG

Table 2.4 Description of constructs and primers used to make constructs

Construct name	Sequence Position on <i>Fgf10</i>	Forward Primer (F)	Reverse Primer (R)	SP1 site
Full	-5853,+502	Full F	Full R	YES
ΔA	-5221,+502	ΔA F	Full R	YES
ΔA-C	-3136,+502	ΔA-C F	Full R	YES
ΔA-D	-2447,+502	ΔA-D F	Full R	YES
ΔA-F	-307,+502	ΔA-F F	Full R	YES
ΔA-G	-63,+502	ΔA-G F	Full R	NO
ΔA-GΔ5'UTR	-63,+72	ΔA-GΔ5'UTRF	Full R	NO
SS-pA3Lucm	-307,+502	SS F	SS R	YES
ΔF	-5853,-2123 -307,+502	FULLSPE F	ΔF R	YES
ΔF-C	-5853,-3593 -307,+502	FULLSPE F	ΔF-C R	YES
ΔF-B	-5853,-4732 -307,+502	FULLSPE F	ΔF-B R	YES
ΔF-A	-5853,-5493 -307,+502	FULLSPE F	ΔF-A R	YES
+SP1	-214,+502	+SP1 F	SS R	YES
-SP1	-171,+502	-SP1 F	SS R	NO
ΔSP1-pA3lucm	-171,+502	ΔA-DΔSP1bglIIF	ΔA-DΔSP1hindIIIR	NO
ΔA-DΔSP1	-2447,-182 -171,+502	ΔA-DΔSP1speF	ΔA-DΔSP1bglIR	NO

Preparation of 3' Deletion Constructs

The region containing the transcription start site (from -307,+502) was amplified by PCR using primers SSF and SSR (Table 2.4). The forward primer incorporated a *BglII* restriction site and the reverse primer incorporated a *HindIII* restriction site (Table 2.3).

Using the Advantage™ 2 PCR kit, samples were preheated to 94 °C for 1 minute, then 5 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds and 69 °C for 1 minute, followed by 30 cycles of 94 °C for 30 seconds, and 69 °C for 1 minute. A final extension at 72 °C for 15 minutes was performed and samples were incubated at 15 °C. PCR products were ligated into pGEM T-easy vector system I, as per manufactures instructions. Inserts were released by restriction digestion using *BglIII* and *HindIII* and ligated, as described in section 2.2.6.1.4, into pA3Lucm that had been digested with the same enzymes. The *Fgf10* transcription start site in pA3Lucm (SS-pA3Lucm) was used to make the 3' deletion constructs. 3' deleted sequences from the *Fgf10* promoter were amplified by PCR using primers Full-Spe F and sequence specific primers detailed in table 2.3. Using the Advantage™ 2 PCR kit, samples were preheated to 94 °C for 1 minute, then 5 cycles of 94 °C for 30 seconds, 51 °C for 30 seconds and 69 °C for 1 minute, followed by 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 69 °C for 1 minute. PCR products were ligated into pGEM T-easy vector system I, as per manufactures instructions. Inserts were released by restriction digestion using *SpeI* and *BamHI* and ligated, as described in section 2.2.6.1.4., into SS-pA3Lucm which had been digested with *NheI* and *BglIII*. 2 µl of each ligation reaction mix was transformed into XL-1 blue cells, as per manufacturers' instructions, sequencing checked the insert was correct, then the plasmid was grown and extracted using a Qiagen® plasmid maxi kit (section 2.2.7.2.).

Preparation of SP1 Deleted Constructs

The SP1 site was 5' deleted and deleted out of ΔA-D by PCR.

5' Deletion of the SP1 Site

The region containing the SP1 site (-214,+502) was amplified by PCR using primers +SP1F and SSR. The region commencing 3' of the SP1 site (-171,+502) was amplified using primers -SPF and SSR. The forward primer incorporated a *SpeI* restriction site

and the reverse primer incorporated a *HindIII* restriction site. Using the Advantage™ 2 PCR kit, samples were preheated to 94 °C for 1 minute, then 5 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds and 69 °C for 1 minute, followed by 30 cycles of 94 °C for 30 seconds, 59 °C for 30 seconds and 69 °C for 1 minute. A final extension at 72 °C for 15 minutes was performed and samples were incubated at 15 °C. PCR products were ligated into pGEM T-easy vector system I, as per manufactures instructions. Inserts were released by restriction digestion using *SpeI* and *HindIII* and ligated, as described in section 2.2.6.1.4., into pA3Lucm which had been digested with *NheI* and *HindIII*. 2 µl of each ligation reaction mix was transformed into XL-1 blue cells, as per manufacturers' instructions, sequencing confirmed the insert was correct, then the plasmid was grown and and extracted using a Qiagen® plasmid maxi kit (section 2.2.7.2.).

Preparation of ΔA-DΔSP1

The region 3' of the SP1 site (-171,+502) was amplified by PCR using primers ΔA-DΔSP1bglIIF and ΔA-DΔSP1hindIIIR. The forward primer incorporated a *BglII* restriction site and the reverse primer incorporated a *HindIII* restriction site. Using the Advantage™ 2 PCR kit, samples were preheated to 94 °C for 1 minute, then 5 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds and 69 °C for 1 minute, followed by 30 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds and 69 °C for 1 minute. A final extension at 72 °C for 15 minutes was performed and samples were incubated at 15 °C. PCR products were ligated into pGEM T-easy vector system I, as per manufactures instructions. Inserts were released by restriction digestion using *BglII* and *HindIII* and ligated, as described in section 2.2.6.1., into pA3Lucm which had been digested with *BglII* and *HindIII*. 2 µl of each ligation reaction mix was transformed into XL-1 blue cells, as per manufacturers' instructions, sequencing confirmed the insert was correct, then the plasmid was grown and and extracted using a Qiagen® plasmid mini kit (section 2.2.7.2.), and named ΔSP1-pA3lucm. The region 5' of the SP1 site (-2447,-183) was amplified by PCR using primers ΔA-DΔSP1speF and ΔA-DΔSP1bglR. The

BglIII site, plus additional nucleotides, replaced the *SpI* site with non-*SpI* sequence. The forward primer incorporated a *SpeI* restriction site and the reverse primer incorporated a *BglIII* restriction site. Using the Advantage™ 2 PCR kit, samples were preheated to 94 °C for 1 minute, then 5 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 69 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 59 °C for 30 seconds and 69 °C for 3 minutes. A final extension at 72 °C for 15 minutes was performed and samples were incubated at 15 °C. PCR products were ligated into pGEM T-easy vector system I, as per manufactures instructions. Inserts were released by restriction digestion using *SpeI* and *BglIII* and ligated, as described in section 2.2.6.1.4., into ΔSP1-pA3lucm which had been digested with *BglIII* and *NheI*. 2 µl of each ligation reaction mix was transformed into XL-1 blue cells, as per manufacturers' instructions, sequencing confirmed the insert was correct, then the plasmid was grown and extracted using a Qiagen® plasmid maxi kit (section 2.2.7.2.)

2.2.7 Plasmid DNA Preparation

2.2.7.1 Plasmid Mini-Preparation

A 5 ml culture of bacteria was grown in LB, with the appropriate antibiotic, at 37 °C in a shaking incubator overnight. 1.5 ml of culture was pelleted in a 1.5 ml eppendorf tube and plasmid DNA was prepared as described in the Qiaprep® mini kit protocol.

2.2.7.2 Plasmid Maxi-Preparation

A 200 ml culture of bacteria was grown in LB using the appropriate antibiotic, overnight at 37 °C in a shaking incubator. The culture was spun down and the remainder LB poured off. The procedure as described in the Qiagen® maxi kit protocol was followed.

2.2.8 Restriction Endonuclease Digestion

Plasmid DNA was digested using the recommended buffer and approximately 2 U enzyme per 1 μ g DNA for 90 minutes. Restriction endonuclease digestion was performed in a water bath at 37 °C.

2.2.9 Agarose Gel Electrophoresis

2.2.9.1 DNA

Plasmid DNA was run on agarose gels of varying percentage and voltage, determined by the best possible resolution of fragments. DNA was loaded into the gels using MBI Fermentas DNA loading dye. UV light was used to visualised ethidium bromide stained DNA and photography was used to record the image.

2.2.9.2. RNA

RNA was run on horizontal 0.6 % (w/v) agarose gels using 1X TAE. MBI Fermentas RNA loading dye was used. RNA was visualised by ethidium bromide staining and photography.

2.2.10 RNase Protection Assay

2.2.10.1 *In Vitro* Transcription of ³²P-Labelled Riboprobes

The plasmid DNA template was linearised with the appropriate restriction endonuclease, to create a 5' overhang. Complete digestion was confirmed by agarose gel

electrophoresis. The linearised template was extracted with an equal volume of phenol:chloroform:iso-amyl alcohol (IAA) (25;24;1) and precipitated by the addition of 0.1 volume 3 M sodium acetate pH 5.2 and 2.5 volume ethanol. The template was incubated at -70°C for 30 minutes then centrifuged at 13 000 rpm for 15 minutes. The pellet was washed in 70 % ethanol, centrifuged at 13 000 rpm for 5 minutes and air-dried for 15 minutes. The linearised plasmid DNA template was re-suspended to a final concentration of $100\text{ ng }\mu\text{l}^{-1}$ in deionised water.

In a reaction volume of $20\text{ }\mu\text{l}$, 100 ng of linearised plasmid DNA template was mixed with transcription reagents to final concentrations of 1X transcription buffer (Promega), 10 mM DTT, 40 U recombinant RNase inhibitor, 500 μM each of ATP, CTP, GTP and 10 μM UTP, 40 μCi $^{32}\text{-P}$ UTP and 15 U of either T3 or T7 DNA polymerase. The reaction mixture was incubated at 37°C for 1 hour. The DNA template was digested by incubation for 20 minutes at 37°C after the addition of 2 U DNase I in DNase buffer to 1 X in a final volume of $50\text{ }\mu\text{l}$. Labelled riboprobe was recovered by column purification (mini Quick Spin RNA Columns). The riboprobe was precipitated with an equal volume of 5 M ammonium acetate and 2.5 volume ethanol at -70°C for 30 minutes. Samples were centrifuged at 13 000 rpm for 20 minutes. The pellet was washed in 70 % ethanol, spun at 13 000 rpm for 5 minutes, air-dried for 15 minutes, and re-suspended in $100\text{ }\mu\text{l}$ of 1 X hybridisation buffer (1 part 5 x hybridisation buffer and 4 parts formamide). The labelled probe could be stored at -20°C for up to 4 weeks.

2.2.10.2 Hybridisation of Labelled Riboprobes

Typically $10\text{ }\mu\text{g}$ of each RNA sample, including one sample of tRNA ($10\text{ }\mu\text{g}$), were precipitated with an equal volume of 5 M ammonium acetate and 2.5 volume ethanol at -70°C for 30 minutes. Samples were centrifuged at 13 000 rpm for 20 minutes. The pellet was washed in 70 % ethanol, spun at 15 000 rpm for 5 minutes, air-dried for 15 minutes, and re-suspended in $10\text{ }\mu\text{l}$ of 1 X hybridisation buffer.

A cocktail mix of probes, including test and internal control probes, was prepared as follows: in a total volume of 10 μ l of hybridisation buffer, 1 μ l of internal control probe and 0.5 μ l of test probe were added per sample. 10 μ l of cocktail was mixed with each RNA and tRNA samples, heated to 95 $^{\circ}$ C for 3 minutes, and incubated overnight at 45 $^{\circ}$ C.

2.2.10.3 RNase Protection Assay

Tubes were briefly centrifuged and placed on ice for 5 minutes. 350 μ l of RNase mixture (1X RNase digestion buffer containing 10 μ l of RNase Cocktail™(Ambion); RnaseA (500 U/ml) and RNase T1 (20000 U/ml) per sample) was mixed into each sample and incubated at 30 $^{\circ}$ C for 45 minutes. Tubes were briefly centrifuged, and 20 μ l 10 % SDS and 5 μ l Proteinase K (10mg ml⁻¹) were added. Tubes were incubated at 37 $^{\circ}$ C for 15 minutes. The protected fragment was extracted with an equal volume of phenol:chloroform: IAA (25;24;1) and precipitated by the addition of 1ml 100 % ethanol at -70 $^{\circ}$ C for 30 minutes. Samples were centrifuged at 13 000 rpm for 20 minutes. The pellet was washed in 70 % ethanol, spun at 15 000 rpm for 5 minutes, air-dried for 15 minutes, and re-suspended in 3 μ l of RNase protection loading dye.

As internal controls, *in vitro* transcribed ³²P-labelled riboprobes and riboprobe size markers were diluted between 1 in 200 and 400 in water and 1 μ l was mixed with 3 μ l of RNase protection loading dye. The cocktail mix of probes was diluted 1 in 40 and 1 μ l mixed with 3 μ l of RNase protection loading dye. All samples were heated to 95 $^{\circ}$ C for 4 minutes and loaded onto pre-run CastAway® Precast Sequencing Gel. Gels were subjected to 70 Watts on a vertical CastAway® Sequencing device for one hour. Gels were fixed in sequencing gel fixative for 10 minutes, washed in dH₂O for 10 minutes, and dried in a Castaway® Gel Dryer. The RNase protection was visualised using Kodak Biomax MS X-ray film, with Amersham Biosciences Hypercassette Intensifying Screens. Hybridised riboprobes were quantified using a phosphoimager and normalised to the internal control.

2.2.11 Western Blot Analysis

2.2.11.1 Protein Extraction

Tissue

Micro-dissected tissue was stored at -70°C until enough was collected to prepare protein. Tissue was thawed in an appropriate volume of tissue protein extraction buffer. Tissue was dissociated by douncing and drawing it through a needle and syringe. Remaining tissue debris was centrifuged and the supernatant containing the protein extract was pipetted off into a new tube.

Cells

Cells were allowed to reach 95 % confluence before being harvested for protein. Cells were rinsed twice in PBS and excess solution was removed. 200 μl of cell protein extraction buffer (per T75 flask) was pipetted on to the cells and incubated on ice for 10 minutes. After 10 minutes the cells were scraped and incubated on ice for a further 10 minutes. Cell lysate was pipetted up and down to further lyse cells and harvested into a 1.5 ml eppendorf. Remaining cell debris was centrifuged and the supernatant containing the protein extract was pipetted off into a new tube.

Protein Quantification

Protein quantification was performed using the Bio-Rad Bradford Assay, as per manufacturers' instructions.

2.2.11.2 Western Electrophoresis, Transfer and Blotting

Appropriate amounts of protein were mixed with final concentrations of 1 X Invitrogen™ NuPAGE™ LDS Sample Buffer and 1 X NuPAGE™ Reducing Agent in a 20 µl volume. Samples were heated for 10 minutes at 70 °C, mixed and loaded on to a 10 % NuPAGE™ Bis-Tris gel with appropriate protein size markers. Western gels were run and blotted following the manufacturers' instructions and using NuPAGE™ Loading Dye, Sample Antioxidant, 1 X MOPS SDS Running Buffer, and 1 X Transfer buffer, and Biosciences Hybond-P Protein Transfer Membrane, all supplied by Invitrogen™.

2.2.11.3 Western Analysis

Transfer membranes were removed from the NuPAGE™ Electrophoresis Apparatus and incubated, at room temperature for one hour on the Platform Shaker STR6, in 2 % (w/v) instant dried semi-skimmed milk dissolved in TBST. The transfer membranes were incubated, at 4 °C overnight, in 2 % (w/v) milk TBST with the appropriate dilution of antibody (see table below). The membranes were washed 3 times for 15 minutes with TBST and incubated for 1 hr with the appropriate secondary AP-conjugated antibody diluted 1 in 20000 in TBST. Membranes were washed in TBST for 30 minutes and subjected to ECF reagent, as per manufacturers' instructions. Protein-antibody conjugates were visualised using a phosphoimager (STORM 860) with a blue chemiluminescence filter.

Table 2.5 Primary and secondary antibodies used in western analysis

Western	Primary antibody in 2 % milk TBST	Secondary in TBST
Androgen Receptor	Anti-androgen receptor 1:200	Anti-rabbit IgG (whole molecule) AP-conjugate 1:20000
Smooth muscle actin	Anti-actin, alpha smooth muscle 1:1000	Anti-mouse IgG (Fab specific) AP-conjugate 1:20000

2.2.12 Immunohistochemistry

2.2.12.1 Paraffin Embedding of Tissue

Micro-dissected tissue or cultured VPs were fixed in 4 % PFA or Bouins fixative for 2 hr on ice. Tissue was stored in 70 % ethanol until processed. Dehydration and further processing was performed on a Leica TP 1050. Paraffin embedding was performed on the Tissue Embedding Center and 5 μ m sections cut using a microtome (Leica RM2135).

2.2.12.2 Tissue Immunohistochemistry

Sections were dewaxed (in xylene) for ten minutes and rehydrated through an ethanol series of 95 %, 80 %, 60 % and 40% for thirty seconds in each. Sections were pressure cooked for 5 minutes in citrate buffer, cooled for 20 minutes and washed twice in TBST on a platform shaker. Sections were blocked in 20 % normal serum (serum of the host the secondary antibody was raised in, see table below) in TBST at room temperature for 45 minutes, followed by an overnight incubation with the required primary antibodies

(see table below) diluted in 20 % normal serum in TBST. Slides were washed three times for 5 minutes in TBST followed by incubation for 1 hr at room temperature in the required IgG-biotinylated secondary antibodies (diluted in 20 % normal serum in TBST, see table). Slides were washed twice for 5 minutes in TBST and twice for 5 minutes in PBS. Sections were then incubated for 3 hours with the required conjugated antibodies and/or streptoavidin-fluorophore complex (diluted in 2 % normal goat serum, see table), followed by an overnight wash in PBS. Nuclei were counterstained using 10 µg ml⁻¹ propidium iodide in PBS for 45 minutes, followed by four washes in PBS for 1 hr. Sections were visualised and imaged using confocal microscopy (Zeiss LSM 500).

Table 2.6 Antibodies used for tissue Immunohistochemistry

Immuno-histochemistry	Antibody and Dilution in 20 % serum	Secondary antibody	FITC/CY5 or streptoavidin complex in 2 % NGS
Androgen Receptor and Smooth Muscle	Anti-Androgen Receptor 1:200	Goat anti-rabbit biotinylated 1:500	Streptoavidin alexafluor 488 1:200
	Anti-actin, α-smooth muscle 1:5000		Goat anti-mouse CY5 1:60
Pan-cytokeratin and BrdU	Anti- cytokeratin (pan) 1:200	Rabbit anti-sheep biotinylated 1:500	Goat anti-mouse CY5 1:60
	Anti-BrdU 1:300		Streptoavidin alexafluor 488 1:200

2.2.12.3 **Cell Immunohistochemistry**

Cells were cultured on glass bottom microwell dishes for 48 hrs at 37 °C in a 5 % CO₂ incubator. Cells were washed twice in PBS, fixed in ice-cold methanol for 5 minutes, and washed twice in PBS. 0.2 % triton in PBS was added for 2 minutes to permeabilise the cells, and cells were washed twice in PBS. Cells were blocked in 20 % normal goat serum in PBS at room temperature for 45 minutes, followed by an overnight incubation with the required primary antibodies (see table below) diluted in 20% normal goat serum in PBS. Cells were washed three times for 5 minutes in PBS followed by incubation for 3 hr at room temperature in the required CY5/FITC-conjugated secondary antibodies (diluted in 2 % normal goat serum in PBS, see table below). Nuclei were counterstained using 10 µg ml⁻¹ propidium iodide in PBS for 30 minutes, followed by four washes in PBS for 1 hr. Cells were visualised and imaged using confocal microscopy (Zeiss LSM 500).

Table 2.7 Antibodies used for cell immunohistochemistry

Immuno-histochemistry	Antibody in 20% normal goat serum	Secondary Antibodies in 2 % NGS
Androgen Receptor and Smooth Muscle	Anti-Androgen Receptor 1:200	Goat anti-rabbit FITC 1:200
	Anti-actin, α-smooth muscle 1:5000	Goat anti-mouse CY5 1:60
Androgen Receptor and β-tubulin	Anti-Androgen receptor 1:200	Goat anti-rabbit FITC 1:200
	Anti-β-tubulin 1:300	Goat anti-mouse CY5 1:60

3 The Isolation and Characterisation of VMP-Derived Primary Cells

3.1 Introduction

The aim of this thesis was to examine the regulation of *Fgf10* transcript levels during prostate organogenesis. A primary cell system from the VMP was established and characterised to study the regulation of *Fgf10* transcripts during prostate organogenesis (Fig. 3.1). Although numerous prostate cell lines exist, the vast majority were derived from diseased prostate epithelium (e.g. BPH, PC-3, LnCaP, TRAMP) and some from adult prostate stromal tissue (e.g. PS-1). *Fgf10* transcripts are expressed in prostatic mesenchyme cells, so a cell type that was derived from mesenchyme capable of inducing prostate development was required. The VMP is a condensed pad of mesenchyme that has been shown to be required for induction of prostate development in males. A female VMP has been identified and prostatic budding can be induced in the female rat VMP in the presence of testosterone (Timms et al., 1995). *Fgf10* transcripts have been localised to the female rat VMP (Thomson and Cunha, 1999). Therefore primary cultures of VMP cells (VMPC) derived from the P0 female VMP were thought to be a potentially good model for studying regulation of *Fgf10* transcript levels.

In order to validate the use of primary VMPC in studies of *Fgf10* gene regulation, it was decided to compare primary VMPC to primary stromal cells from non-*Fgf10* expressing cells to identify any differences in VMP and URS markers between cell types. Also it was hoped to compare the activity of *Fgf10* promoter constructs in primary cells that do not express *Fgf10* transcripts but were derived from a similar origin to primary VMPC. As the VMP is part of neonatal reproductive tract primary VMPC were compared to a cell type derived from another part of the tract. It

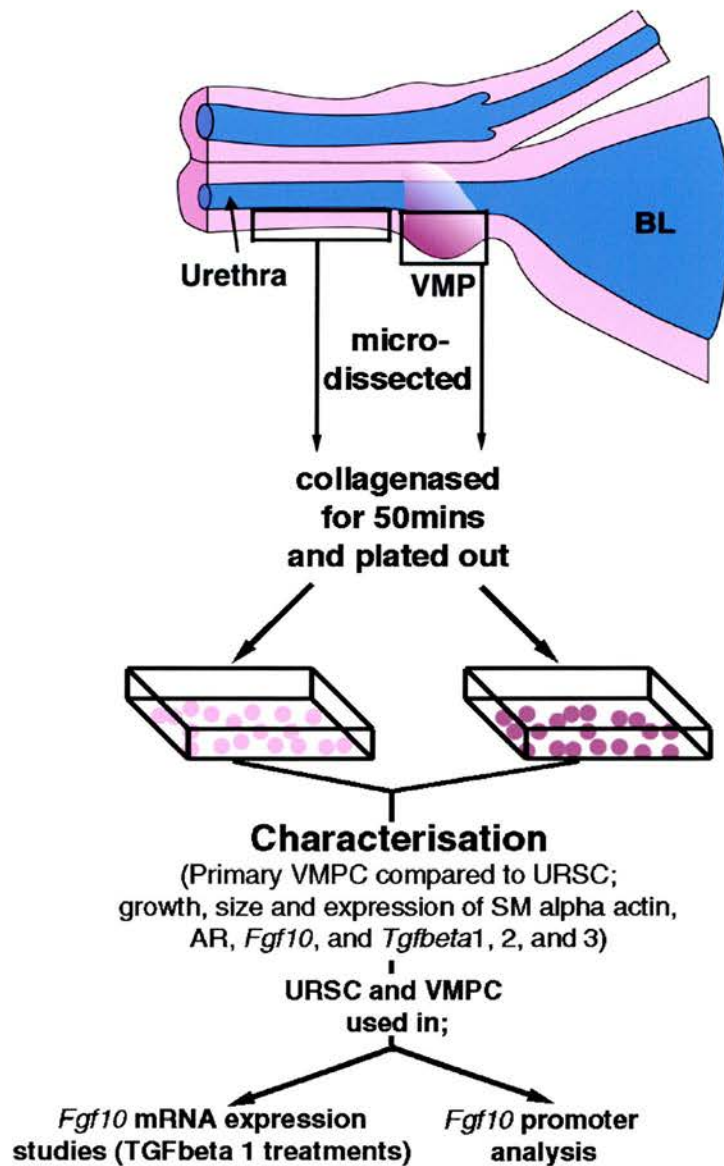


Fig. 3.1 Diagrammatic representation of the derivation, characterisation and analysis of primary mesenchymal prostatic cells

Primary prostatic cells were derived from either P0 rat urethra stromal (URS) or ventral mesenchymal pad (VMP). Cells were derived by collagenase-disassociation of microdissected URS and VMP. Primary VMP cells (VMPC) and URS cells (URSC) were plated out in T75 flasks and were passaged a maximum of 4 times during which they were characterised. Cells were treated with androgens and/or TGFbeta1 to study the regulation of endogenous *Fgf10* mRNA. Cells were also used in transfection studies to identify *Fgf10* promoter elements.

has been shown that non-VMP urethral stroma (URS) (shown on Fig. 3.1) does not express *Fgf10* transcripts, by *in situ* hybridisation (Thomson and Cunha, 1999), so it was decided to compare primary VMPC to primary URS cells (URSC). Primary cultures of VMPC and URSC were established as described (Fig. 3.1), and passaged a maximum of four times. These primary cell types were fully characterised to establish whether they maintained the features of the tissues from which they were derived, and hence were a good model for the study of *Fgf10* transcript regulation. The following parameters were examined: cell size, growth rate, responsiveness of growth to testosterone, and the expression of markers normally expressed in the organ rudiments from which the cell types were derived (AR, SM α actin, *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3*, and *Fgf10*).

3.2 Results

3.2.1 Primary VMPC and URSC Size Differences

Initial observations of primary VMPC and URSC by bright field microscopy indicated there might be a difference in cell size between passage numbers and cell types. Numerous reports have indicated that an increase in cell size may be a result of stress or senescence (Hayflick and Moorhead, 1961; Rubin, 1997; Schneider and Mitsui, 1976). Enzymatic dispersion has been reported to severely stress vertebrate cells (Rubin, 1997). It has also been suggested that there is an intrinsic fixed limit to the number of divisions that normal vertebrate cells can undergo before they senesce or terminally differentiate (Hayflick and Moorhead, 1961). Also late passage primary cultures have a significantly larger volume than early passage cells (Schneider and Mitsui, 1976). Thus it was decided to measure the size of primary VMPC and URSC at different passage numbers.

Primary VMPC and URSC were fixed at low confluence (approximately 40 %), and their surface area measured by brightfield microscopy (Fig. 3.2). Primary VMPC were

examined over three passages and URSC were examined over four passages, as these were the maximum number of passages that could be performed with these cell types. As there is an intrinsic fixed limit to the number of divisions that normal vertebrate cells (i.e. untransformed) can undergo before they senesce or terminally differentiate, and that cells at late passage cultures are larger (i.e. older and more senescent or terminally differentiated), this data itself suggested that primary VMPC senesce or terminally differentiate faster than primary URSC. The same number of primary VMPC and URSC were passaged suggesting that the differences in senescence is not due to the differences in number of cells in each experiment i.e. a different number of cell divisions to confluence. However the amount of VMP tissue compared to the amount of URS tissue used for dissociation was hard to keep the same. Hence differences in cell senescence could have been due to different amounts of tissue being dissociated and therefore different numbers of cells plated out. If different amounts of VMP and URS tissue were being used then after dissociation the cells should reach confluence at different times, however this was not observed suggesting similar number of cells were being dissociated and plated out. This suggests that the differences in VMPC and URSC senescence is probably due to differences obtain from the where the tissue was derived and not differences in the number of cells dissociated and plated out.

After the first passage the average primary VMPC size was $3604 \mu\text{m}^2$ (Fig 3.2B). After passage number two VMPC size ($8210 \mu\text{m}^2$) had increased two-fold. The third and final passage caused a further small increase in cell size to $9506 \mu\text{m}^2$. After the first passage the average URSC size was $2323 \mu\text{m}^2$, which slightly increased after the second passage to $2756 \mu\text{m}^2$. A third passage resulted in an approximately two-fold increase to $5293 \mu\text{m}^2$, and the fourth and final passage resulted in a 1.7-fold increase in URSC size to $8991 \mu\text{m}^2$.

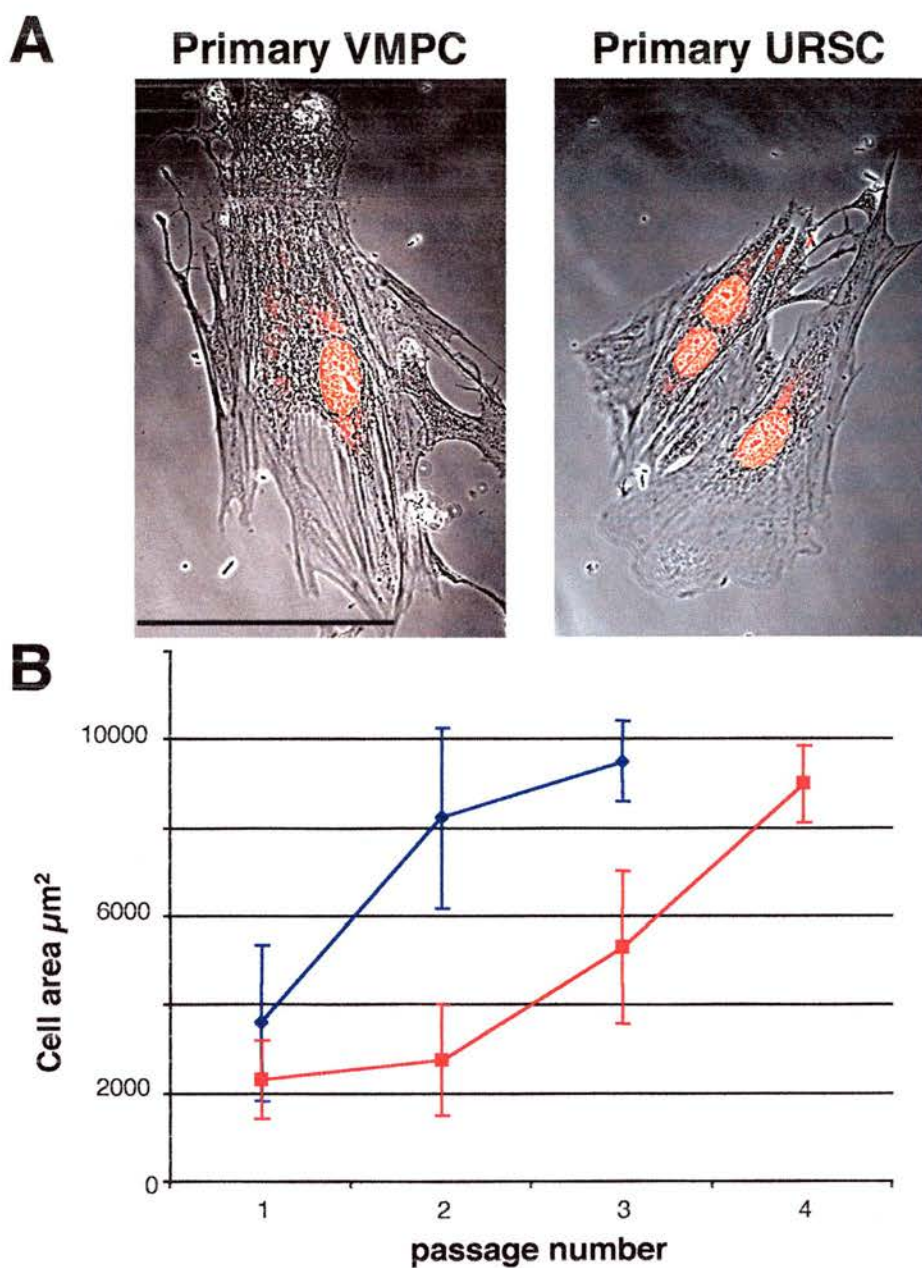


Fig. 3.2 Primary VMPC and URSC size differences

Cells were fixed and their nuclei stained with propidium iodide before being imaged and measured using phase contrast microscopy. If the cells borders were indistinguishable, cell area represents the total surface area of a group of cells divided by the number of nuclei present. Panel A shows primary VMPC and URSC at passage number two (scale bar = 100μm) and panel B represents cell size at different passage times (VMPC; blue line and URSC; red line). VMPC and URSC size increases with passage number but VMPC become larger earlier in all experiments (n=3). Error bars are standard error to the mean (SEM).

At passage number one the average VMPC size was 1.5 X larger than the average URSC size (Fig 3.2B). By passage number two VMPC were visibly larger than URSC (Fig 3.2A), which was confirmed by measurement to a value of 3-fold. At passage number three VMPC were 2 X larger than URSC but after four passages, URSC were of similar size to VMPC at passage number three.

These data show rapid increase in primary VMPC size at passage number two, suggesting that these cells had begun to senesce. Primary URSC increased in size slower than VMPC, indicating that they senesce more slowly. This data demonstrated that there was a consistent difference in cell size between primary VMPC and URSC suggesting that primary VMPC senesce more quickly than primary URSC.

3.2.2 The Growth Rate of Primary VMPC and URSC

Another marker of cell stress and senescence is a decline in growth rate with passage number (Rubin, 1997). It was observed that at higher passage numbers primary VMPC took longer to become confluent than URSC. This was confirmed by comparing cell growth rate, for both cell types at each passage (Fig 3.3). Cell growth rate was defined as the fold change in number of cells between day two and day six of culture, and represents the total number of cells still alive after the four days in culture.

The growth rate of primary URSC did not change between passage number one and two, but by passage three the fold change in number of cells had decreased from 11-fold to 7-fold. On passage number four the fold change in number of cells had further decreased to just 3-fold. On passage number one primary VMPC had increased in number by 10-fold. By passage number two there was only a 3-fold change in cell number, and on passage number three primary VMPC did not increase in cell number over the four-day culture.

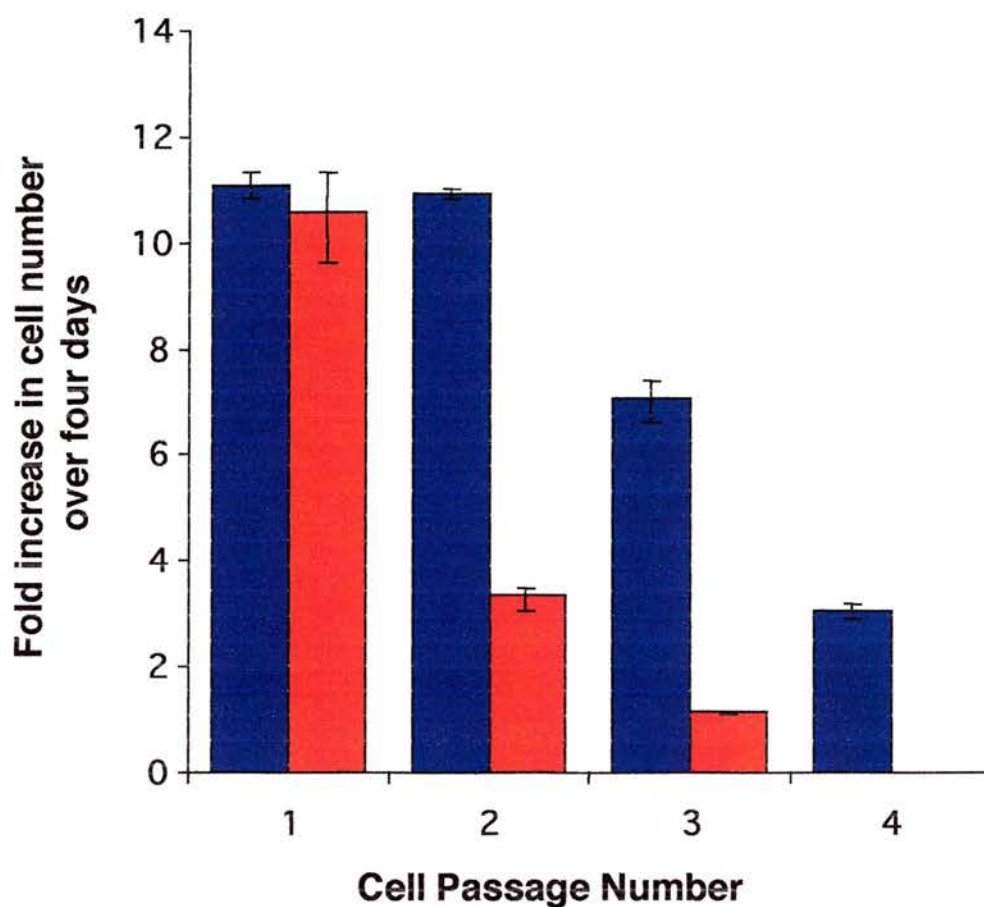


Fig. 3.3 Changes in cell growth rate

Cell number was counted for primary VMPC (red bar) and URSC (blue bar) after each passage. The number of cells in two T75 flasks of each cell type were counted on day two and six of culture and the fold increase in cell number determined. Cells were counted in duplicate and the graph represents the average of three experiments. On passage number one VMPC number increased by ten-fold, but this decreased to three-fold by passage number two, over the four-day culture. By passage three VMPC number did not change over the four-day culture. URSC number increased by over ten-fold on passage number one and two, seven-fold on passage number three and three-fold on passage number four, over the four day culture period. Error bars are SEM.

Primary URSC and VMPC growth rate was similar on passage number one. On passage number two URSC grew over 3.5 X quicker than VMPC, and on passage number three URSC grew 7 X quicker than VMPC. On passage number four URSC growth rate was similar to VMPC growth rate on passage number two.

It has been speculated that a decline in cell growth rate with passage number is a marker of cell senescence, which implies that primary VMPC start to senesce on passage number two and primary URSC start to senesce on passage number three. This data is comparable to primary VMPC and URSC size differences i.e. as cell size increases, cell growth rate decreases, which are signs of cell senescence or terminal differentiation (Rubin, 1997).

3.2.3 Effect of Testosterone on Primary VMPC and URSC number

Growth and development of the prostate are dependent on androgens. Data from primary cultures of prostate stromal and epithelial cells, and organ transplantation experiments suggest that epithelial cell proliferation is not directly regulated by androgens, but is growth responsive to androgen-stimulated stromal cells (Chang and Chung, 1989; Gao et al., 2001). Also, androgens have been shown to inhibit the growth rate of primary epithelial cells cultured without stroma (Chang and Chung, 1989; Nishi et al., 1988), and promote cell proliferation in prostatic stromal cells (Chang and Chung, 1989; Gerdes et al., 1996). The effect of testosterone on the growth rate of primary VMPC and URSC was investigated (n=3).

Primary VMPC and URSC were treated with testosterone and counted on day 2, 4 and 6 of culture, and the number of cells was compared to the number of cells in untreated cultures. Cells were cultured in DMEM both in the presence (Fig 3.4A) and absence (Fig. 3.4B) of 10 % FBS and/or testosterone. It was demonstrated that testosterone did

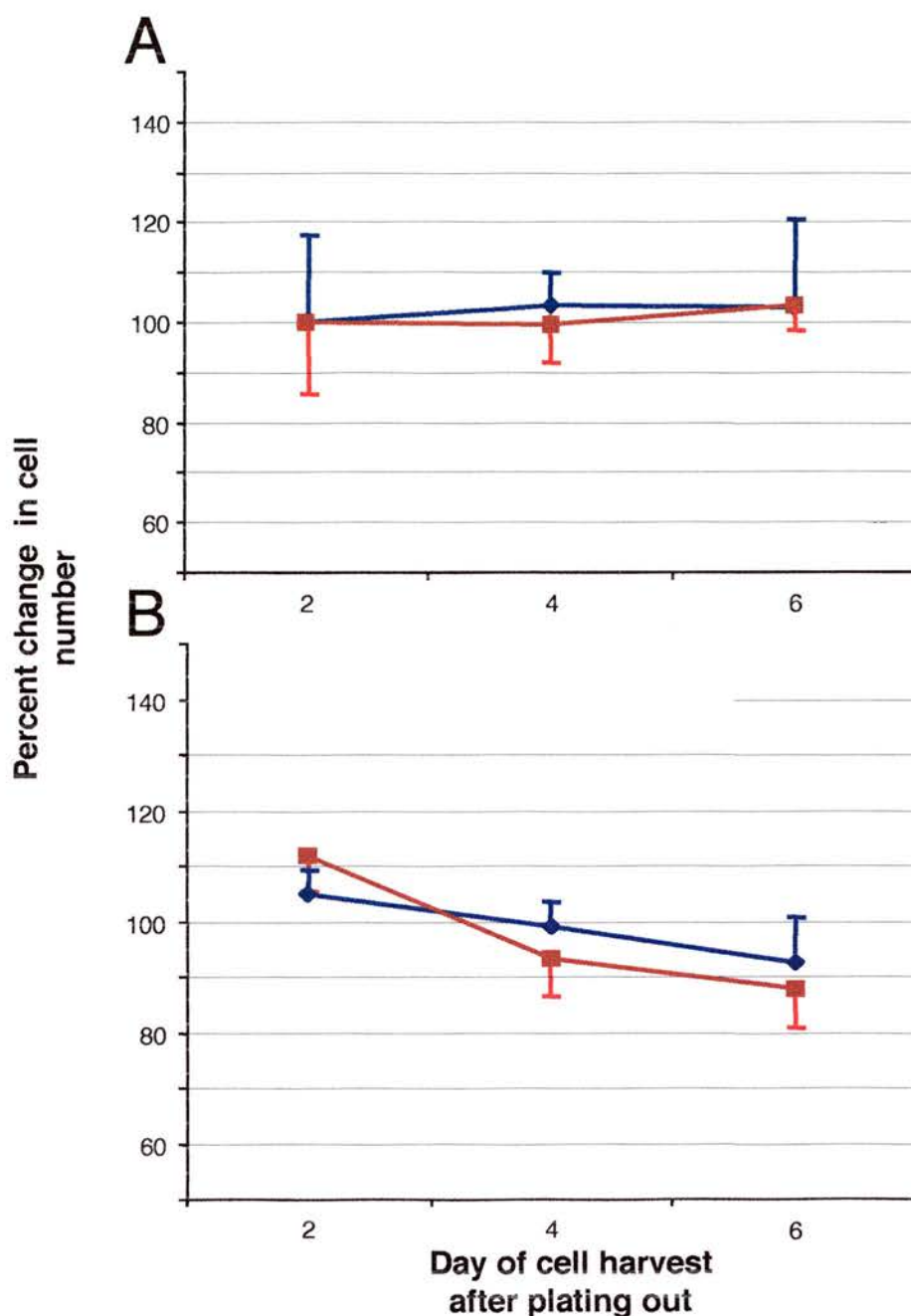


Fig. 3.4 The effect of testosterone on cell growth rate

The effect of testosterone on the growth rate of primary VMPC (blue line) and URSC (red line) after the second passage. Panel A, cells grown in the presence of 10 % FCS. Panel B, cells grown under serum free conditions. The X-axis represents the day after plating that cells were counted. The Y-axis shows the percentage change in cell number for testosterone treated cells relative to vehicle-treated cells. Cell counts were performed in duplicate and the graphs represent an average of three experiments. Testosterone caused no significant change in primary VMPC or URSC number. Error bars are SEM.

not significantly alter primary VMPC or URSC number compared to untreated cells in either the presence or absence of serum.

3.2.4 Comparison of AR and SM α Actin Expression between Female UGTs, and Primary VMPC and URSC

3.2.4.1 AR Expression

Early in prostate development, androgen/AR interactions that occur in stromal cells induce growth and development of prostatic epithelial cells (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980; Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985), and AR expression in the mesenchyme is required for the development of the prostate (Cunha and Chung, 1981; Cunha and Lung, 1978). *Fgf10* transcript levels were postulated as being regulated by testosterone in prostate cells and as primary VMPC were derived to examine the regulation *Fgf10* transcript levels we thought it necessary that primary VMPC express AR. Also we wanted to establish a cell type that represented inductive prostatic stromal tissue and as androgen/AR is essential for this process, again we felt it necessary that primary VMPC express AR. AR protein expression in the female UGT was examined and compared to AR expression in primary VMPC and URSC, by immunohistochemistry and western blotting (n=3).

AR was expressed in both epithelial and stromal cells of the P0 female rat UGT (Fig. 3.5B and 3.5D). AR appeared to be expressed at a higher level in the VMP than UR (Fig. 3.6A-C).

AR expression was observed in all primary VMPC (Fig. 3.7A) and URSC (Fig 3.7C), by immunohistochemistry. The amount of AR expressed was examined, in protein samples prepared from cell lysates at each passage number and compared to protein sampled prepared from microdissected VMP and UR, by western blotting (Fig 3.8A). Primary

VMPC and URSC expressed AR at each passage number, and the amount of protein expressed was similar between cell types and passage numbers. The amount of AR in primary VMPC and URSC was comparable to the amount expressed in the UR rudiment. However, a higher expression of AR was observed in the VMP rudiment than either cell type.

3.2.4.2 SM α Actin Expression

The expression pattern of SM α actin was examined in P0 female rat UGTs and in primary VMPC and URSC, by immunohistochemistry (n=3) and western blotting (n=3).

Immunohistochemistry showed SM α actin was expressed in a distinct layer surrounding the urethra along the P0 female rat UGT (Fig 3.5A and D). The smooth muscle layer formed an obvious barrier between the VMP and UR (Fig. 3.6A, B and C), and VMP cells in vivo (Fig 3.5A) are SM α actin negative. To determine whether micro-dissection of the VMP (for collagenase-disassociation) resulted in any SM layer being included, a western blot was performed on protein samples prepared from micro-dissected VMP and URs (Fig. 3.9A). The VMP rudiment protein expressed SM α actin, demonstrating micro-dissected VMPs had some smooth muscle layer attached, and that micro-dissection of the VMP was not precise. Only a small layer of SM would represent a significant proportion of the dissected VMP, and as the epithelium was not removed from the UR the proportion of SM was under represented in UR protein compare to if it was URS protein alone. This may explain why the amount of SM α actin was similar between micro-dissected samples of UR and VMP.

SM α actin expression was observed in all primary VMPC (Fig. 3.7A) and URSC (Fig. 3.7C), by immunohistochemistry. The amount of SM α actin was examined, in protein samples prepared from cell lysates at three passages by western blotting (Fig. 3.9A).

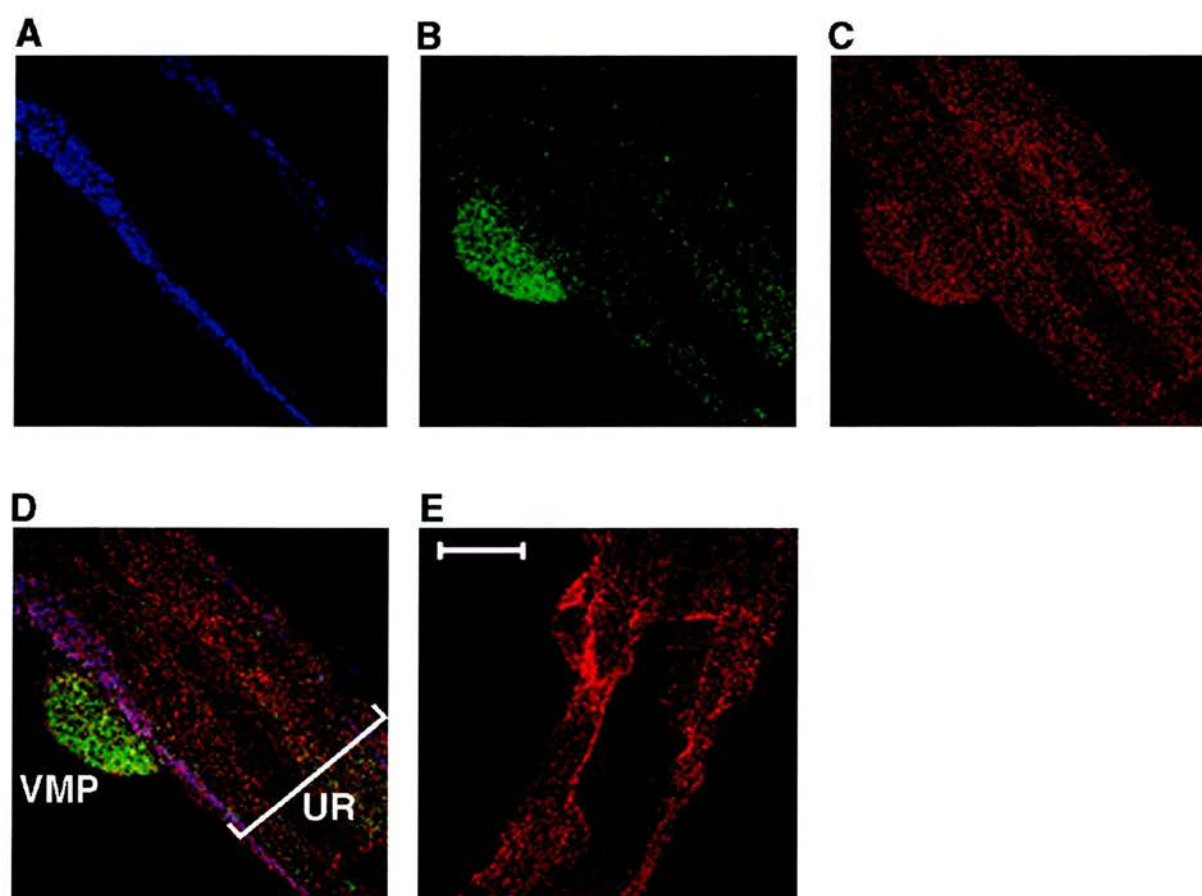


Fig. 3.5 AR and smooth muscle alpha actin expression in the female UGT
 Immunohistochemistry for androgen receptor (AR) and smooth muscle alpha actin (SM alpha actin) on the female urogenital sinus (UGS). SM alpha actin is shown in blue (panel A), AR in green (panel B), nuclear staining in red (propidium iodide) (panel C) and the three colours merged is shown in panel D. Primary antibodies were not added to panel E. In the UGS AR expression is higher in the VMP than urethra (UR), and SM alpha actin is expressed in UR stromal cells but not the VMP (n=3). The scale bar shown in panel E represents 200 μ m.

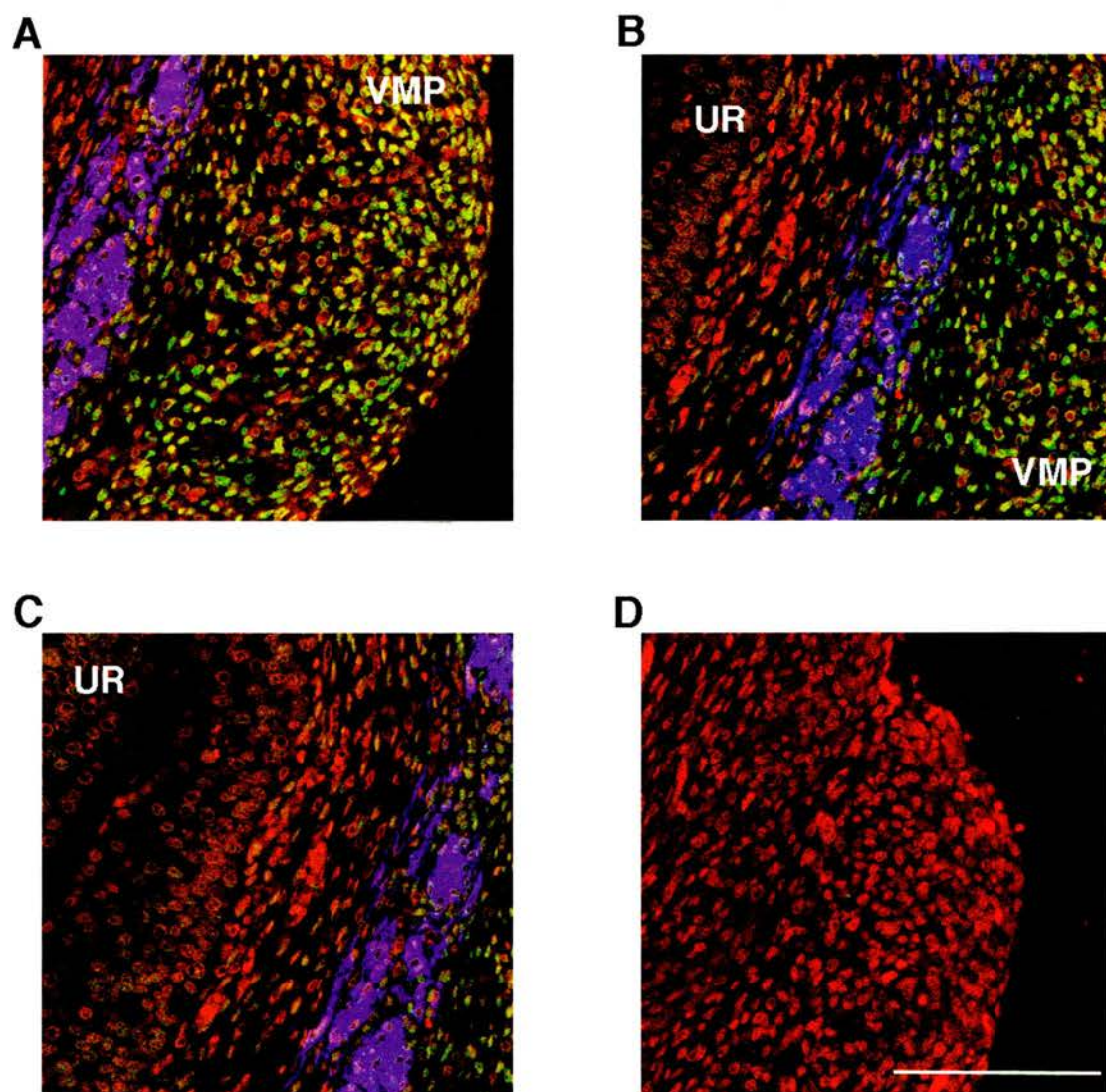


Fig. 3.6 AR and smooth muscle expression in the VMP and UR

Immunohistochemistry showing AR and SM alpha actin expression in the VMP (panels A and B) and urethra (UR, panels B and C) at x40 magnification (scale bar 100um). AR is green (yellow staining represents AR localised to the nucleus), SM alpha actin blue, and nuclear staining is red. Primary antibodies were not added to panel D. The figure compares UR and VMP AR stromal expression and demonstrates that stromal SM alpha actin expressing cells separate the VMP from UR. Panel B shows a clear difference in AR expression between the UR and VMP stroma, more AR is expressed in the VMP than UR (n=3).

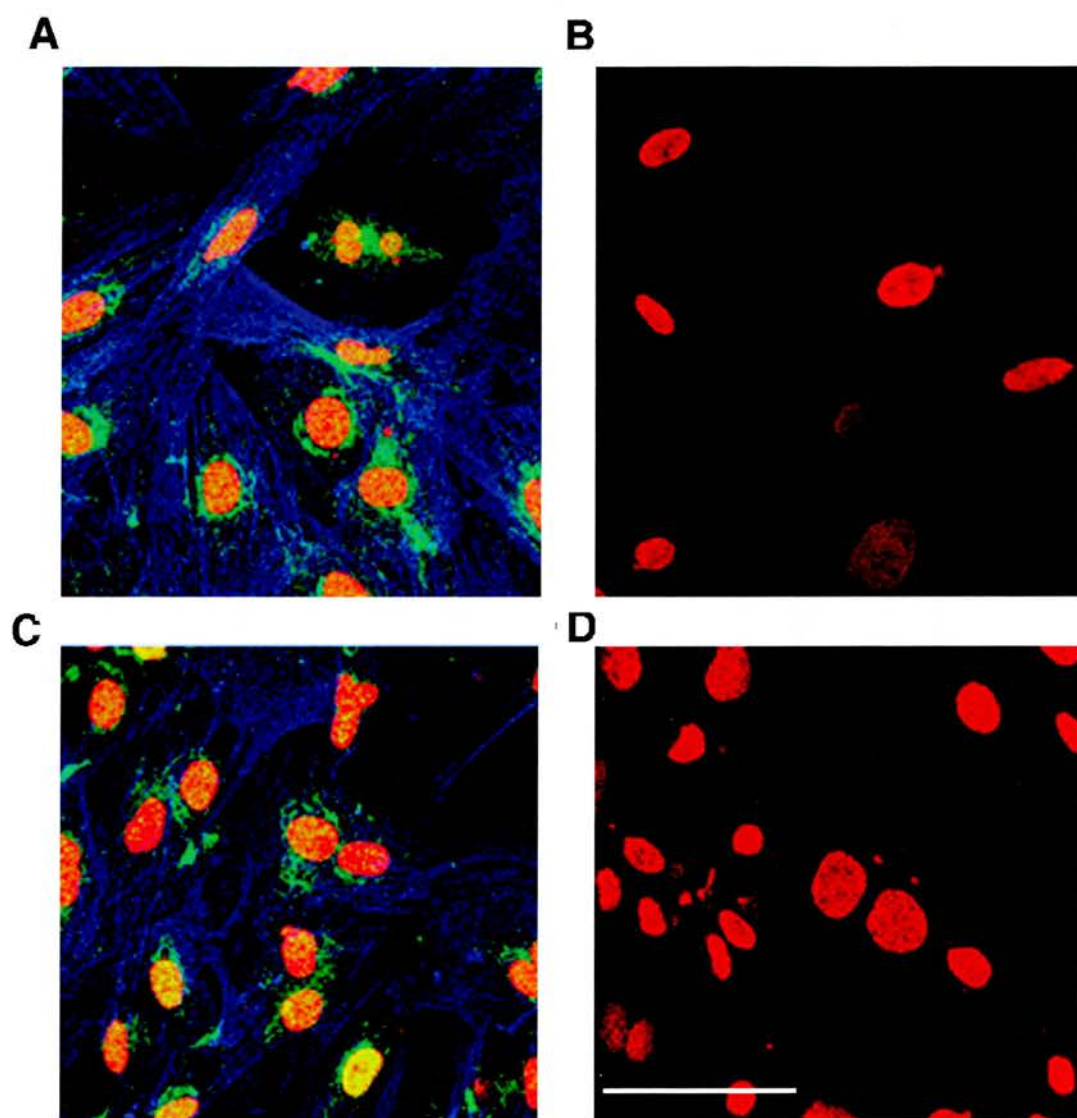


Fig. 3.7 AR and smooth muscle alpha actin expression in primary VMPC and URSC
 Immunohistochemistry for AR and SM alpha actin on primary VMP (panel A) and URS (panel C) cells. SM alpha actin is blue, AR is green (yellow when localised to the nucleus), and nuclear staining (propidium iodide) is red. Primary antibodies were not added to VMPC (panel B) and to URSC (panel D). AR and SM alpha actin was expressed in all cells examined (n=3). The scale bar in panel D represents 100 μ m.

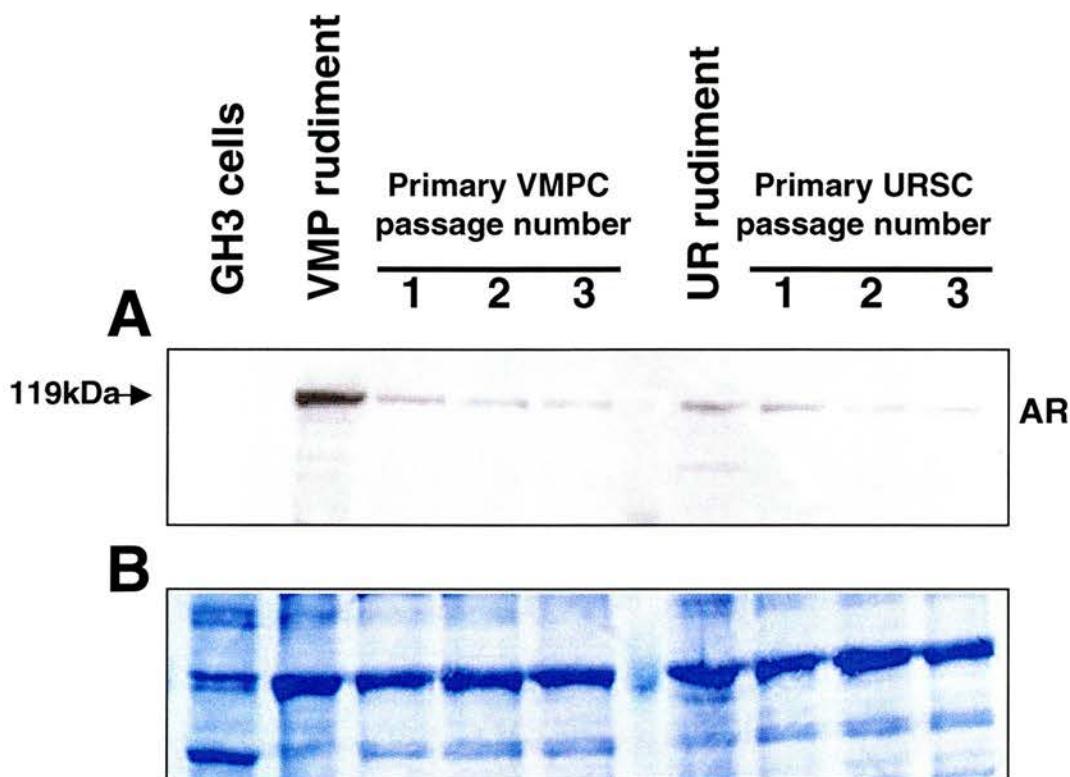


Fig. 3.8 AR expression in the VMP, UR, primary VMPC and primary URSC
 Western blot for AR (98KDa but approximately 119KDa when glycosylated) on UR and VMP organ rudiments and their respective primary cell protein of different passage numbers. 15µg of protein was loaded per sample. Panel A shows a western analysis and panel B shows the coomassie stain of the blot to show consistent protein loading. AR expression was highest in the VMP organ rudiment, the primary VMPC and URSC expressed AR at similar levels throughout each passage (n=3).

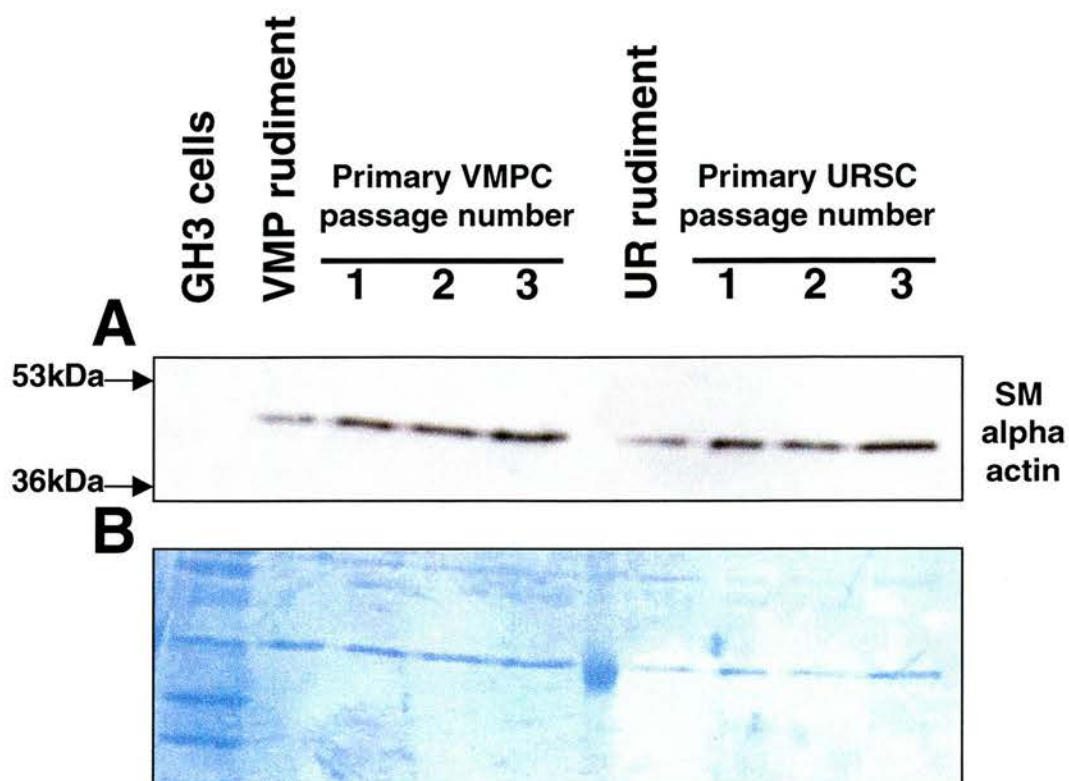


Fig. 3.9 SM alpha actin expression in the VMP, UR, primary VMPC and URSC
 Western blot for SM alpha actin (42KDa) on UR and VMP organ rudiments and their respective primary cell protein at different passage numbers. 1 μ g of protein was loaded per sample. Panel A shows a western analysis and panel B shows the coomassie stain of the blot to show consistent protein loading. SM alpha actin was expressed at similar levels between cell types and between passage numbers (n=3).

Primary VMPC and URSC expressed SM α actin at every passage and the amount was similar between cell types and passage numbers. The amount of SM α actin protein in VMPC and URSC was comparable to the amount in the VMP and UR rudiments. This result demonstrates firstly that both cell types were derived from stroma (Janssen et al., 2000; Sensibar et al., 1999), and secondly that SM α actin protein is expressed in both primary VMPC and URSC when cultured *in vitro*.

3.2.5 Analysis of *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* Transcript Levels

TGFBetas have been shown to be expressed in the prostate (Perry et al., 1997; Timme et al., 1995) and may regulate growth during development and adulthood (Itoh et al., 1998a; Kyprianou and Isaacs, 1989). Hence, next it was decided to investigate the expression pattern of *Tgfbeta* transcripts in primary cells and organ rudiments. Experiments were designed to examine the expression pattern of *Tgfbeta* transcripts to determine if similar levels of *Tgfbeta* transcripts are expressed in primary VMPC and URSC and their respective organ rudiment. *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcript levels were analysed in female prostatic organ rudiments and primary VMPC and URSC by RNase protection assay.

DNA templates for the synthesis of *Tgfbeta1*, 2, and 3 riboprobes were cloned by RT PCR using P0 VP cDNA. Primers (chapter 2.2.5.) were designed using GenBank-published cDNA sequences (Accession numbers; *Tgfbeta1*, NM_021578; *Tgfbeta2*, AF135598; *Tgfbeta3*, NM_013174). Protected products for *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* were 261nts, 245 nts, and 324 nts, respectively. Female UGTs were micro-dissected either for RNA preparation or for collagenase-disassociation for cells (Fig. 3.1). Cells were plated out and RNA harvested after passage number one. RNA from VMP and UR rudiments and primary VMPC and URSC were hybridised with ³²P-radiolabelled riboprobes for *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* and cyclophilin.

Transcript levels were quantified using a phosphoimager and normalised to cyclophilin. In the VMP *Tgfbeta* transcript levels were approximately 1.3-fold (*Tgfbeta1*), 1.8-fold (*Tgfbeta2*) and 5.6-fold (*Tgfbeta3*) higher than in the UR (Fig. 3.10A). In primary VMPC, *Tgfbeta* transcript levels were approximately 1.5-fold (*Tgfbeta1*), 2.8-fold (*Tgfbeta2*) and 4.8-fold (*Tgfbeta3*) higher than in primary URSC (figure 3.10B). The expression pattern of *Tgfbeta* isoforms in organ rudiments is maintained in their respective primary cells.

3.2.6 Analysis of *Fgf10* Transcript Levels

As FGF10 plays an important role in the growth of the prostate (Thomson and Cunha, 1999), *Fgf10* transcript levels in VMP and UR organ rudiments were compared to primary VMPC and URSC by RNase protection assay.

A DNA template for the rat *Fgf10* riboprobe was previously published (Thomson and Cunha, 1999). Female UGTs were micro-dissected either for RNA preparation or for collagenase-disassociation for cells (Fig. 3.1). Primary cells were plated out and RNA harvested after passage number one, two and three. RNA from VMP and UR rudiments and primary VMPC and URSC at different passage numbers were hybridised with ³²P-radiolabelled riboprobes for *Fgf10* and cyclophilin. Transcript levels were quantified using a phosphoimager and normalised to cyclophilin. The same P0 VP sample was included in each RNase protection assay so that different experiments could be compared. *Fgf10* transcript levels in the VMP were 14 times higher than those expressed in the UR and 4 times higher than levels in VPs (Fig. 3.11A). *Fgf10* transcript levels in the VMP were 4 times higher than those expressed in primary VMPC and URSC (Fig. 3.11B). UR organ rudiments expressed similar levels *Fgf10* transcripts to primary VMPC and URSC. However, primary URSC only expressed *Fgf10* transcripts in passage numbers one and two and expression was lost after passage three.

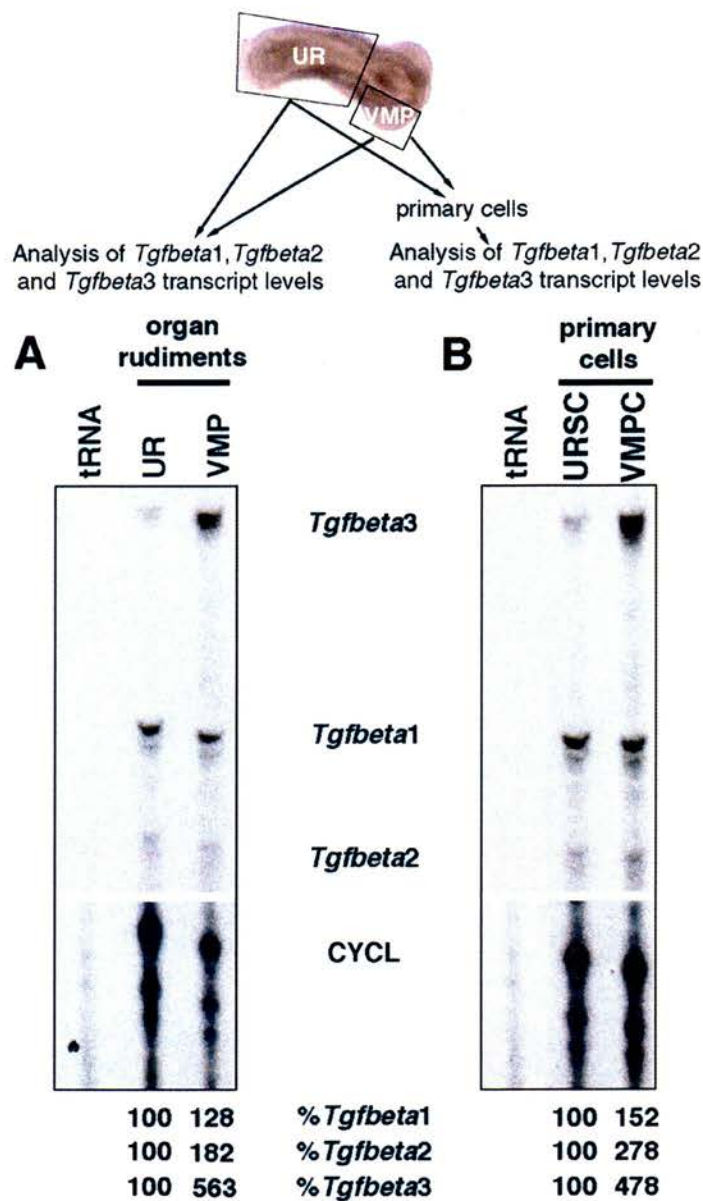


Fig. 3.10 *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* expression in the female UGT and primary VMPC and URSC

Analysis of *Tgfbeta1*, 2 and 3 transcript levels in female organ rudiments and their respective primary cells by RNase protection. Transcript levels were quantified using a phosphoimager and normalised to cyclophilin (CYCL). The numbers below the figure represent the average transcript abundance of *Tgfbeta1*, 2 and 3 relative to UR rudiment (panel A, n=2) or primary URSC (panel B, n=4). Panel A. *Tgfbeta1*, 2 and 3 transcripts levels were highest in the VMP. Panel B. *Tgfbeta1*, 2 and 3 transcript levels were highest in primary VMPC at passage two reflecting levels in the VMP rudiment.

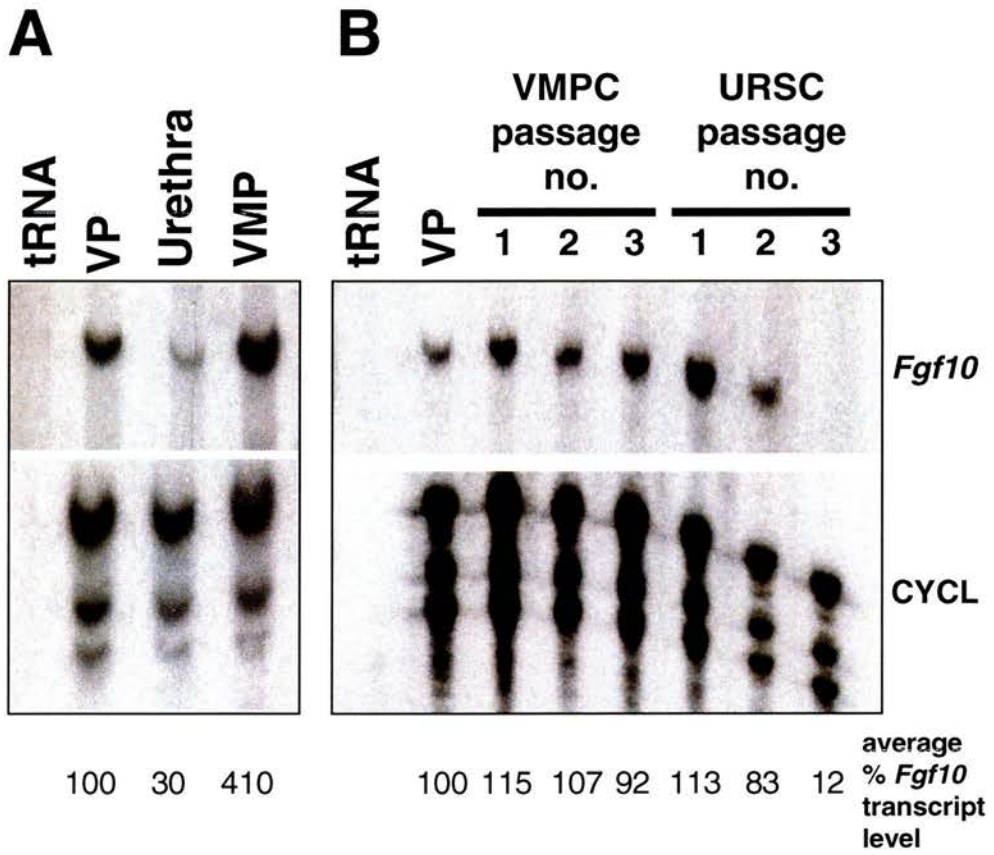
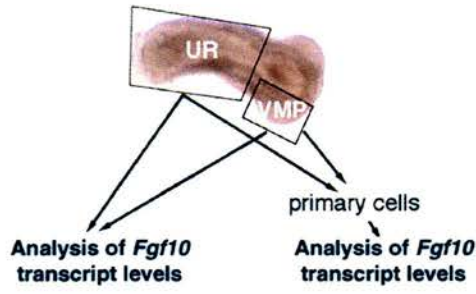


Fig. 3.11 *Fgf10* transcript expression in the female UGT and primary VMPC and URSC

Comparison of *Fgf10* transcript levels in female UR and VMP organ rudiments (panel A) and their respective primary cells (panel B) at different passage numbers. Loading was normalised using cyclophilin (CYCL). The numbers below panel A and B represent the average transcript abundance of *Fgf10* relative to levels in P0 VP (n=3). *In-vitro* *Fgf10* transcripts are expressed mainly in the VMP, fourteen fold higher than the UR. At passage numbers one and two primary VMPC and URSC both express *Fgf10* transcripts but URSC *Fgf10* transcript levels decline at passage three. Passage number three represents approximately three weeks in culture.

3.3 Summary of Results

Primary VMPC and URSC became larger and their growth-rate slower with increased passage number. These changes were consistent with cellular senescence (Rubin, 1997), and this data suggested that primary VMPC and URSC senesced after passage three and four, respectively. Also primary VMPC and URSC showed no change in growth rate when cultured with testosterone suggesting testosterone does not directly regulate stromal cell growth.

Markers normally expressed in organ rudiments were also present in their respective cell types, and some differences between primary VMPC and URSC were identified. In particular SM α actin expression was detected in all primary VMPC but not in the VMP by immunohistochemistry, suggesting that the cells differentiated and that there were some changes in primary VMPC in culture. However, the most striking similarity between organ rudiment and cell type was the expression pattern of *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* transcripts. *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* gene expression was maintained between organ rudiment and cell type, suggesting that primary VMPC and URSC were of different lineage and represented the organ rudiment from which they were derived. *Fgf10* transcripts were expressed at low levels in the UR, by RNase protection assay, despite previously not being detectable by *in situ* hybridisation (Thomson and Cunha, 1999). *Fgf10* transcripts were also expressed in primary URSC at similar levels to primary VMPC. *Fgf10* transcript levels in primary VMPC were maintained throughout their three passages, while at passage number three *Fgf10* transcript levels in primary URSC were decreased. It appeared that primary VMPC maintained similarities to the VMP rudiment and primary URSC maintained similarities to the URS so it was decided that at low passage numbers primary VMPC were a good system in which to study the regulation of *Fgf10* gene expression.

4 Regulation of *Fgf10* Transcript Levels in Cells

4.1 Introduction

This chapter describes experiments to examine regulation of *Fgf10* transcript levels in primary VMPC (characterised in chapter 3) and two cell lines, PS-1 and NIH3T3 cells. Previous investigations into regulation of *Fgf10* gene expression demonstrated that both androgens (Lu et al., 1999) and TGFbeta1 (Beer et al., 1997; Lebeche et al., 1999) might play a role in regulating *Fgf10* transcript levels. To address these possible interactions experiments were designed to determine if *Fgf10* transcript levels were regulated in prostate and non-prostate cells cultured in the presence of testosterone or TGFbeta1, and if testosterone could repress TGFbeta1 action.

Three types of cells were cultured, two of which were derived from rat prostate rudiments (PS-1 cells (Gerdes et al., 1996) and primary VMPC) and the third (NIH3T3) was derived from embryonic mouse fibroblasts (Muller et al., 1984). PS-1 cells and primary VMPC were cultured because they have been characterised and shown to express *Fgf10* transcripts. NIH3T3 cells were cultured as TGFbeta1 down-regulated *Fgf10* transcripts in 3T3 cell types (Beer et al., 1997); therefore the NIH3T3 cell line was a control for TGFbeta1 action.

4.2 Results

4.2.1 AR and *Tgfbeta* Receptor Type I Expression in NIH3T3, PS-1 and Primary VMPC Cell Types

Since these studies were designed to examine testosterone and TGFbeta1 regulation of *Fgf10* gene expression it was important to confirm the expression of AR and *Tgfbeta* receptor type I.

4.2.1.1 AR expression

AR expression was examined by immunohistochemistry (Fig. 4.1A) and western blotting (Fig. 4.1B). Immunohistochemistry showed AR immunoreactivity in PS-1 cells and primary VMPC (also shown in Fig. 3.6, 3.7 and 3.8). AR was localised both to the cytoplasm and nucleus of PS-1 cells and primary VMPC. Some staining was seen in NIH3T3 cells, but was not thought to represent AR (discussed in detail below). A 119 KDa band, previously shown to represent AR, was visualised in PS-1 and primary VMPC protein, by western blotting. No full length AR was detectable in NIH3T3 cell protein by western blotting.

4.2.1.2 *Tgfbeta* Receptor Type I

Primary VMPC, NIH3T3, and PS-1 cells were cultured in the presence and absence of TGFbeta1 for 48 hrs. RNA was prepared from the cells and RT-PCR was performed (Fig. 4.2). Rat ovary cDNA was used as a positive control for *Tgfbeta* receptor type I expression. The quality of the cDNA was verified by RT-PCR for HPRT. *Tgfbeta* receptor type I was expressed in cells when cultured in the presence of TGFbeta1. No *Tgfbeta* receptor type I expression was detected in primary VMPC cultured in the

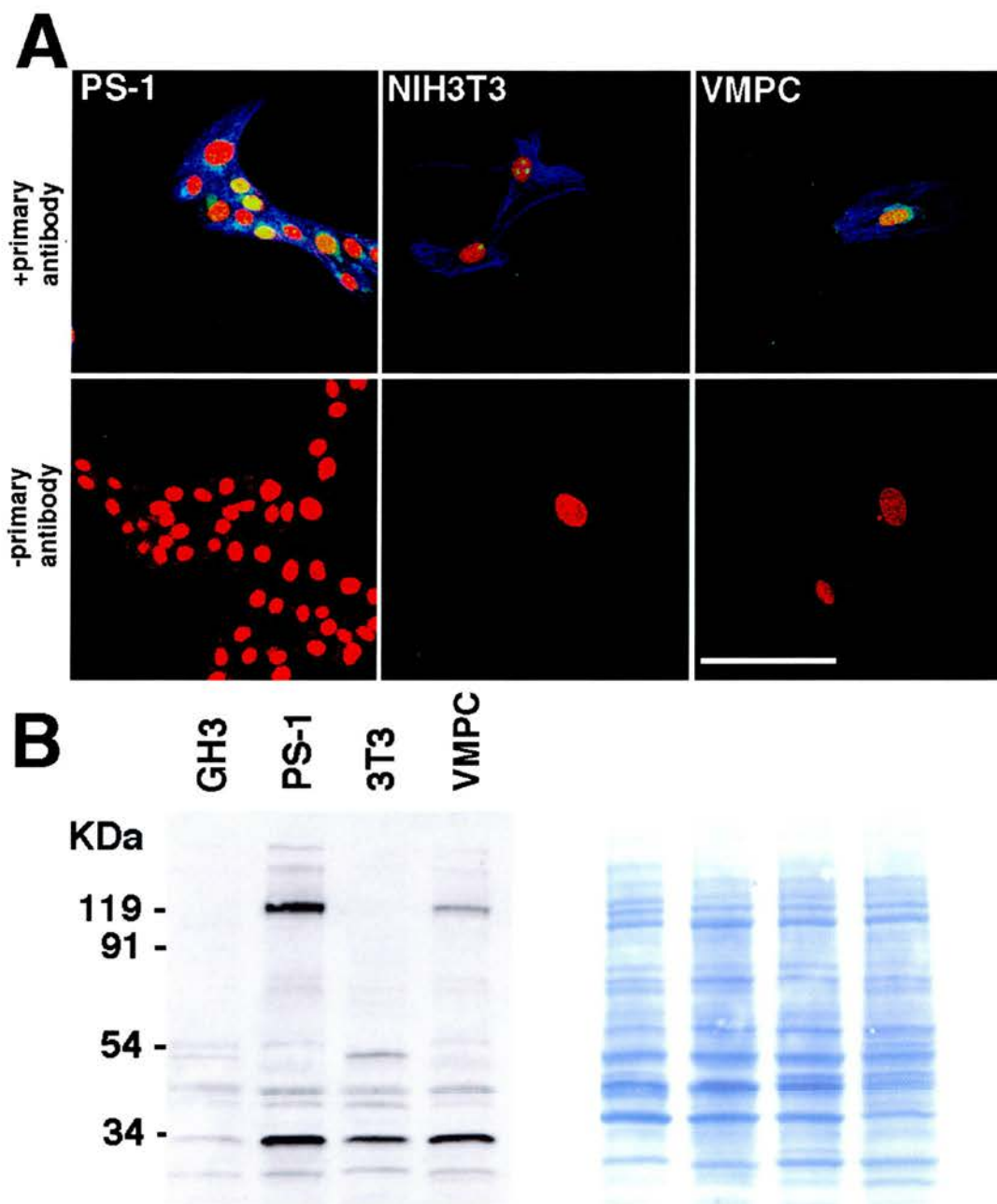


Fig. 4.1 AR expression in primary VMPC, NIH3T3 and PS-1 cells
 Immunohistochemistry (panel A) and western blot (panel B) for AR expression in PS-1, NIH3T3 and primary VMPC. Images were captured by confocal microscopy at x40 (scale bar =100 μ m). Panel A - AR is green (yellow when co-localised with the nucleus), beta-tubulin is blue and the nucleus is red (propidium iodide). AR expression was demonstrated in PS-1 and primary VMPC but not in NIH3T3 cells. Panel B - Western blot showing AR (98KDa but approximately 119KDa when glycosylated) expression in PS-1 and primary VMPC but not NIH3T3 cells (left side), loading was confirmed by coomassie staining (right side).

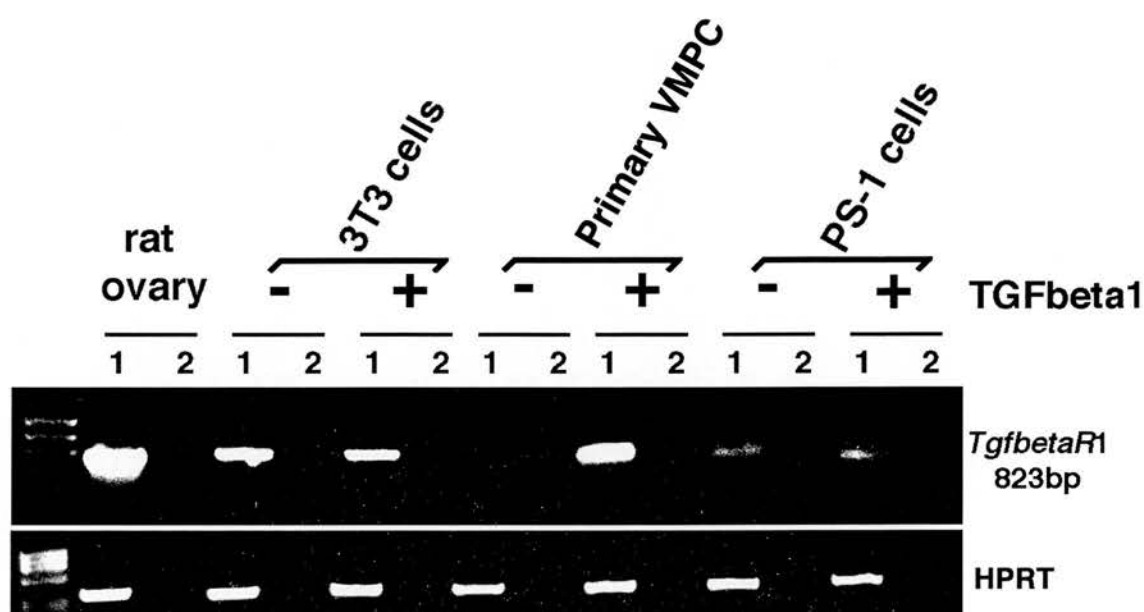


Fig. 4.2 *Tgfbeta* receptor 1 (*TgfbetaR1*) expression in NIH-3T3, VMPC and PS-1 cells.

cDNA was prepared from NIH-3T3, VMPC and PS-1 cells cultured in the presence (+) or absence (-) of TGFbeta1 for 48 hrs (1 = +reverse transcriptase (RT), 2 = -RT), and PCR for *TgfbetaR1* and HPRT was performed. Panel A. NIH-3T3 and PS-1 cells express *TgfbetaR1* independent of TGFbeta1 treatment. *TgfbetaR1* expression in primary VMPC is dependent on TGFbeta1 treatment (n=3). Panel B. PCR for HPRT was performed on the same cDNA samples as *TgfbetaR1* PCR to demonstrate the presence of cDNA.

absence of TGFbeta1, suggesting that primary VMPC were the only cell type to show TGFbeta1 regulation of the *Tgfbeta receptor type I*.

4.2.2 Regulation of *Fgf10* Transcript Levels in Cells Cultured with TGFbeta1 or Testosterone for 48 hrs

The long-term effect (48 hrs) of TGFbeta1 and testosterone on *Fgf10* transcript levels in cells was examined. Each cell type was cultured in the presence or absence of serum and with or without TGFbeta1 or testosterone for 48 hrs. After 48 hrs cells were harvested for RNA. RNA from cells were hybridised with ³²P-radiolabelled riboprobes for *Fgf10* and cyclophilin.

4.2.2.1 NIH3T3 cells

There was no significant change in *Fgf10* transcripts levels in NIH3T3 cells cultured in the presence of serum compared to cells cultured in the absence of serum (Fig. 4.3). A two-fold increase of *Fgf10* transcripts levels was observed in cells cultured in the presence of testosterone and serum, but this was not observed in the absence of serum. A four-fold reduction in *Fgf10* transcripts levels was observed in cells cultured in the presence of TGFbeta1 in the absence of serum, but the regulation of *Fgf10* gene expression was not observed in the presence of serum.

4.2.2.2 Primary VMPC

A consistent two-fold increase in *Fgf10* transcript levels was observed in primary VMPC grown in the absence of serum (Fig. 4.4). *Fgf10* transcript levels were significantly higher than transcript levels in the P0 VP, when primary VMPC were cultured in the absence of serum. *Fgf10* transcript levels in primary VMPC cultured in

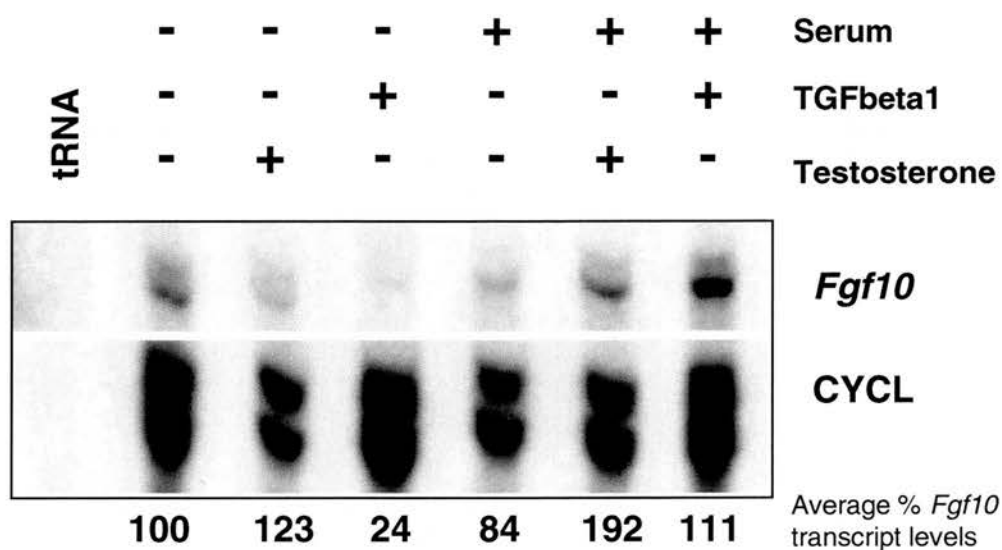


Fig. 4.3 Analysis of *Fgf10* transcript levels in NIH3T3 cells by RNase protection assay

NIH3T3 cells were cultured in the presence or absence of serum and/or testosterone (10^{-8} M) or TGFbeta1 (5ng/ml) for 48hrs. *Fgf10* transcript levels were normalised to cyclophilin (CYCL). The numbers below the figure represent the average transcript abundance of *Fgf10* relative to levels on serum free treated cells (n=3). *Fgf10* transcript levels in cells cultured in the presence of serum and testosterone increased by two fold. Cells grown in the presence of TGFbeta1 but absence of serum resulted in a four fold decrease in *Fgf10* transcript levels.

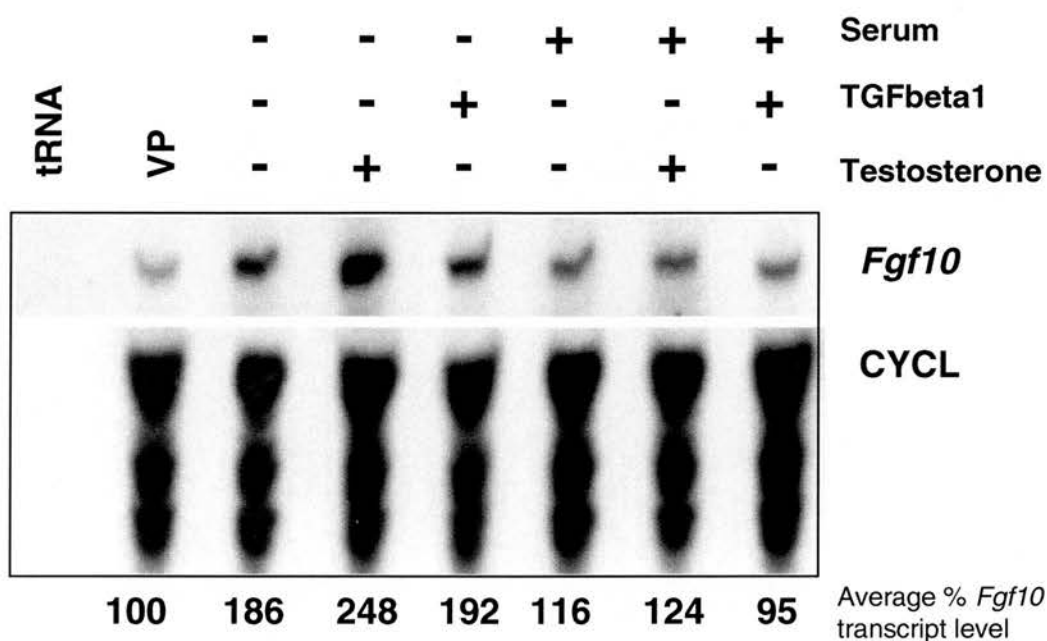


Fig. 4.4 Analysis of *Fgf10* transcript levels in primary VMPC, by RNase protection assay.

Primary VMPC cells were cultured in the presence or absence of serum and/or testosterone (10^{-8} M) or TGFbeta1 (5ng/ml) for 48hrs. *Fgf10* transcript levels were normalised to cyclophilin (CYCL). The numbers below the figure represent the average transcript abundance of *Fgf10* relative to levels in P0 VP (n=3). *Fgf10* transcript levels in VMP cells cultured in the presence of testosterone or TGFbeta1 were not significantly changed. A consistent increase of *Fgf10* transcripts levels was observed in cells grown in the absence of serum.

the presence of testosterone or TGFbeta1 were not significantly changed from untreated cells, when examined at 48 hours.

4.2.2.3 PS-1 cells

The same P0 VP sample was included in each RPA so that *Fgf10* transcript levels could be compared between PS-1 cell and primary VMPC treatments. *Fgf10* transcript levels in PS-1 cells were less than half the levels expressed in the VP (Fig. 4.5). *Fgf10* transcript levels were not significantly affected in the presence or absence of serum and/or testosterone, or TGFbeta1 in PS-1 cells.

Fgf10 transcript levels were significantly lower in PS-1 cells than in the VP, and by extrapolation this suggested that *Fgf10* transcripts are expressed at a lower level in PS-1 cells than primary VMPC. *Fgf10* transcript levels in PS-1 cells was considered too low to examine *Fgf10* gene regulation by TGFbeta1, as TGFbeta1 represses *Fgf10* transcript levels and RNase protection assay were not sensitive enough to detect any significant decreases in *Fgf10* gene expression in PS-1 cells. Hence studies with PS-1 cells were not continued.

4.2.3 Regulation of *Fgf10* transcripts in NIH3T3 cells and primary VMPC cultured with TGFbeta1 for 1, 3, 7, and 48 hrs

Down-regulation of *Fgf10* transcript levels by TGFbeta1 has been shown to be rapid and reversible in 3T3 cells (Beer et al., 1997), so next an experiment was designed to determine if a similar regulation of *Fgf10* gene expression was observed in prostate cells. NIH3T3 cells and primary VMPC were cultured in the absence of serum and presence of TGFbeta1 for 1, 3, 7 and 48hrs. As controls primary VMPC were cultured

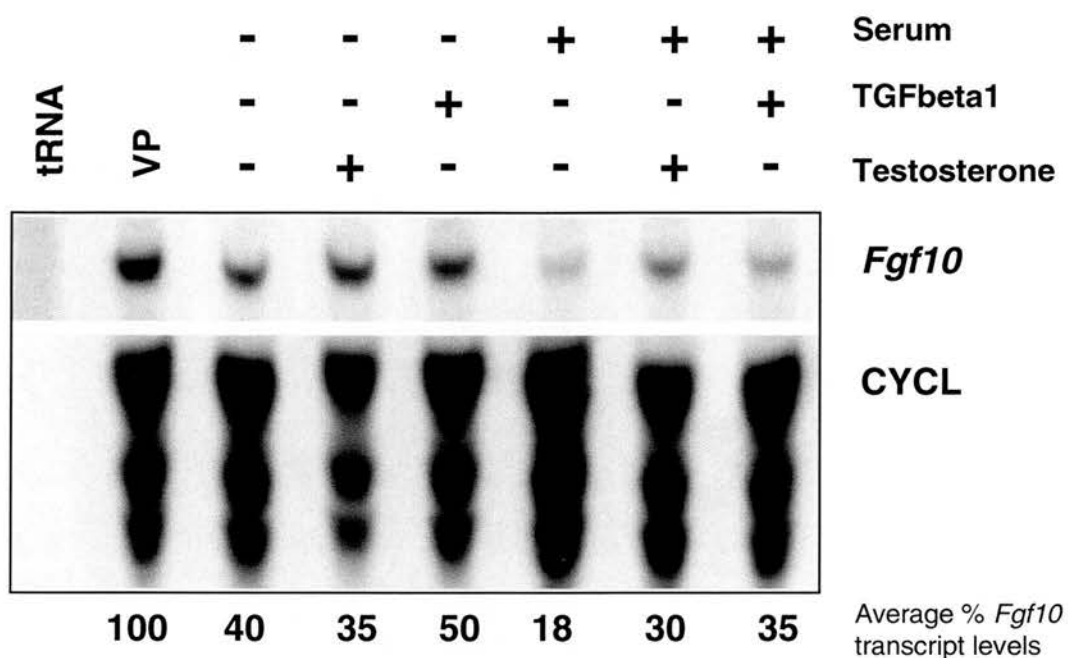


Fig. 4.5 Analysis of *Fgf10* transcript levels in PS-1 cells by RNase protection assay.

PS-1 cells were cultured in the presence or absence of serum and/or testosterone (10^{-8} M) or TGFbeta1 (5ng/ml) for 48hrs. *Fgf10* transcript levels were normalised to cyclophilin (CYCL). The numbers below the figure represent the average transcript abundance of *Fgf10* relative to levels in P0 VP (n=3). PS-1 *Fgf10* transcript levels were not significantly affected in the presence or absence of serum and/or testosterone or TGFbeta1.

in the absence of TGFbeta1 for 1 and 48 hours. RNase protection assay was used to quantify any changes in *Fgf10* transcript levels.

4.2.3.1 NIH3T3 cells

Fgf10 transcript levels, in cells cultured with TGFbeta1, decreased two-fold after one hour, four-fold after three hours and five-fold after seven hours compared to untreated cells (Fig. 4.6). After a 48 hr TGFbeta1 treatment *Fgf10* transcript levels had not recovered to previous levels in NIH3T3 cells, which agrees with a previous experiment performed over 48 hours (Fig. 4.3).

4.2.3.2 Primary VMPC

Fgf10 transcript levels, in primary VMPC cultured with TGFbeta1, showed little change after one hour, but decreased fourteen-fold after three hours, and four-fold after seven hours compared to untreated cells (Fig. 4.7). This repression was reversible and *Fgf10* transcript levels returned to levels observed in untreated cells after 48 hrs. Again these results agreed with previous results in experiments performed over 48 hrs (Fig. 4.4).

4.2.4 Testosterone represses TGFbeta1 inhibition of *Fgf10* transcript levels

Ligand-bound AR has been shown to inhibit TGFbeta transcriptional responses (Chipuk et al., 2002) so next we wanted to determine if the down-regulation of *Fgf10* transcript levels by TGFbeta1 could be repressed by the addition of testosterone. Chipuk *et al.* demonstrated the inhibition of TGFbeta transcriptional responses over 48 hours. However there was no repression of *Fgf10* transcripts by TGFbeta1 after 48 hours in cultured primary VMPC (Fig. 4.7). The greatest repression was observed over three hours (Fig. 4.7) but it was not known whether the effect observed by Chipuk *et al.* would

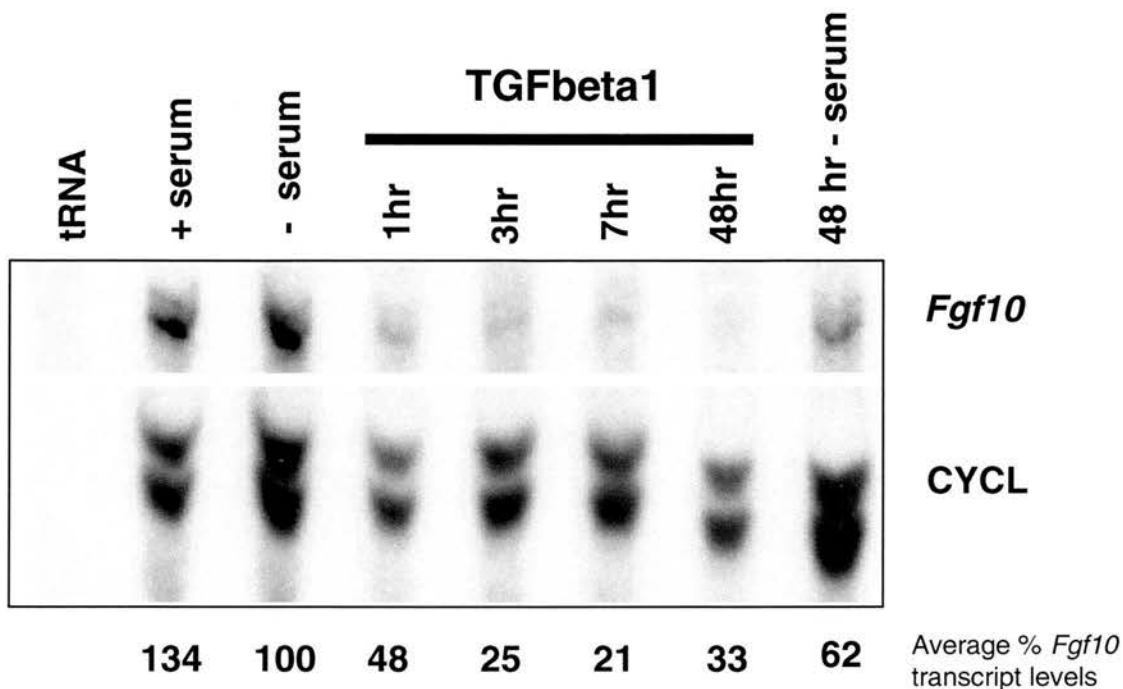


Fig. 4.6 Analysis of *Fgf10* transcript levels in NIH3T3 cells cultured with TGFbeta1, by RNase protection.
 Cells were cultured in serum free media for 1 and 48hrs and with TGFbeta1(5ng/ml) for 1, 3, 7 and 48hrs. *Fgf10* transcript levels were normalised to cyclophilin (CYCL). The numbers below the figure represents the average transcript abundance of *Fgf10* relative to levels in cells grown in the absence of serum (n=3). Cells cultured in the presence of TGFbeta1 resulted in a five fold down regulation of *Fgf10* transcript levels after seven hours. *Fgf10* transcript levels did not recover to previous levels after a 48 hour TGFbeta1 treatment.

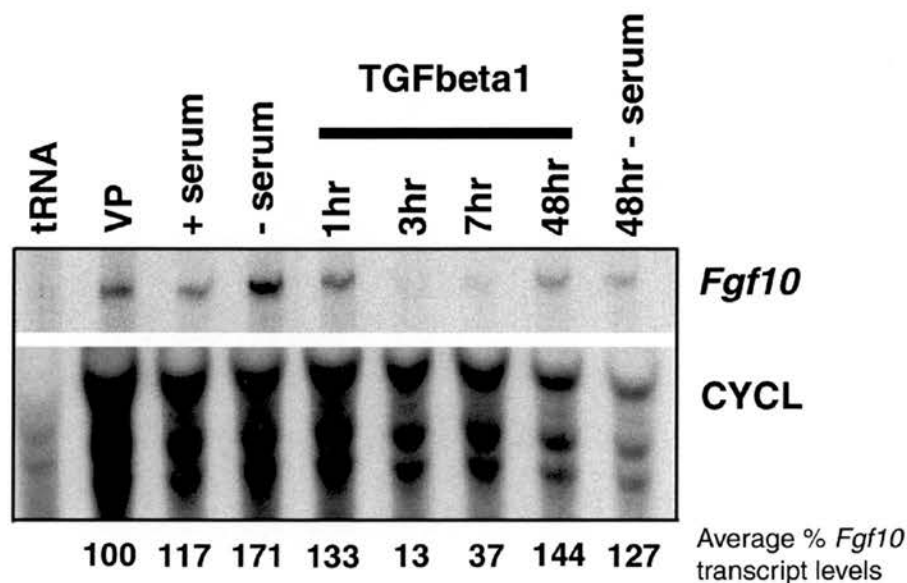


Fig. 4.7 Analysis of *Fgf10* transcript levels in primary VMPC cultured with TGFbeta1, by RNase protection.

Cells were cultured in serum free media for 1 and 48 hrs and with TGFbeta1 (5ng/ml) for 1, 3, 7, and 48 hrs. *Fgf10* transcript levels were normalised to cyclophilin (CYCL). The numbers below the figure represent the average transcript abundance of *Fgf10* relative to levels in P0 VP (n=3). *Fgf10* transcript levels in primary VMPC cultured for three hours in the presence of TGFbeta1 decreased by thirteen fold. *Fgf10* transcript levels in cells cultured for 48 hrs in the presence of TGFbeta1 were similar to those found in untreated cells.

occur over this short period of time. Hence it was decided that these experiments would be performed over seven hours, as this was the longest time point examined that *Fgf10* transcripts were repressed. Primary VMPC were cultured for 24 hrs in the absence of serum then cells were cultured in the presence or absence of TGFbeta1 and/or testosterone for 7hrs. RNA was prepared and hybridised with ³²P radiolabelled *Fgf10* and cyclophilin riboprobes.

No change in *Fgf10* transcript levels was observed after culture for 7 hours with testosterone (Fig. 4.8). A three-fold reduction in *Fgf10* transcript levels was observed in primary VMPC after culture with TGFbeta1, which agrees with a previous experiment performed over the same period of time (Fig. 4.7). There was no change in *Fgf10* transcript levels in cells cultured with TGFbeta1 and testosterone, suggesting that testosterone could inhibit TGFbeta-mediated repression of *Fgf10* gene expression over seven hours. This study was performed towards the end of my experiments and does raise the question of whether testosterone inhibits TGFbeta-mediated repression of *Fgf10* transcript levels over three hours. In retrospect, the experiment should have been performed over three hours as well as seven hours.

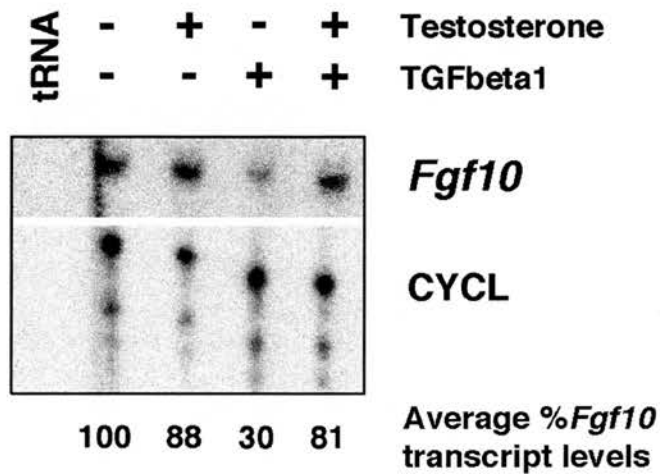


Fig. 4.8 Analysis of *Fgf10* transcript levels in primary VMPC cultured with TGFbeta1 and/or testosterone, by RNase protection.

Cells were cultured in serum free media for 24 hrs then cultured in presence of testosterone (10^{-8} M) and/or TGFbeta1 (5ng/ml) for 7 hours. Cell RNA were hybridised with 32 P-radiolabelled *Fgf10* and cyclophilin (CYCL) probes. *Fgf10* transcript levels were normalised to CYCL. The numbers below the figure represent the average transcript abundance of *Fgf10* relative to levels in untreated cells (n=3). VMPC *Fgf10* transcript levels were not significantly affected when cultured for seven hours in the presence of testosterone. However testosterone inhibited the TGFbeta1 down-regulation of *Fgf10* transcripts over this time period.

Table 4.1 Changes in *Fgf10* transcript levels culture in the presence of serum

Cell type	Treatment (average percentage change in <i>Fgf10</i> transcript levels compared to untreated cells)	
	TGFbeta1 (48 hrs)+/-SEM	Testosterone (48 hrs) +/-SEM
PS-1	194 +/-6.7	166 +/-8.7
NIH3T3	133+/-9.6	229+/-34.5
Primary VMPC	82+/-9.6	107+/-31.5

Table 4.2 Changes in *Fgf10* transcript levels cultured in the absence of serum

Cell type	Treatment (average percentage change in <i>Fgf10</i> transcript levels compared to untreated cells)						
	TGFbeta1 +/-SEM				Testosterone +/-SEM		Testosterone and TGFbeta1 +/-SEM
	1hr	3hr	7hr	48hr	7hr	48hr	7hr
PS-1	-----	-----	-----	125+/-15.8	-----	88+/-20.4	-----
NIH3T3	48+/-14.9	25+/-8.0	21+/-6.8	29+/-10.3	-----	123+/-31.4	-----
Primary VMPC	77+/-24.1	8+/-11.0	22+/-17.7	94+/-29.2	88+/-10.1	133+/-31.5	81+/-12.4

4.3 Summary of Results

Experiments were performed to determine whether TGFbeta1 and/or testosterone regulated *Fgf10* transcript levels in cells. Primary VMPC, NIH3T3, and PS-1 cells were cultured in the presence and absence of TGFbeta1 and/or testosterone for varying periods of time, and levels of *Fgf10* transcripts were quantified by RNase protection assay (shown in Tables 4.1 and 4.2).

Data presented here suggests that there is no direct regulation of *Fgf10* transcript levels by androgens over 48 hrs in PS-1 cells and primary VMPC. However, androgens did increase *Fgf10* transcript levels in NIH3T3 cells. AR expression was observed in primary VMPC and PS-1 cells but not in NIH3T3 cells. The data presented here is

highly suggestive that NIH3T3 cells do not express AR even though some immunohistochemical staining was observed. AR expression was restricted to small areas within the nucleus in NIH3T3 cells while AR is usually located diffusely around the cytoplasm and in the nucleus suggesting the staining observed in NIH3T3 cells was not AR. To confirm this western blotting was performed, and no full length AR was observed, indicative of a lack of AR. Bands were observed that were smaller than the typical AR and may represent a truncated form of AR. Overall this data suggested that ligand-bound AR did not directly affect *Fgf10* transcript levels in NIH3T3 cells. Also, as NIH3T3 cells were derived from embryonic mouse fibroblasts, the observed changes in *Fgf10* transcript levels were probably not representative of a prostate-like response. Our data in PS-1 cells and primary VMPC agrees with data published by Thomson and Cunha, 1999, suggesting that *Fgf10* gene expression is not regulated by androgens, but does not agree with data published by Lu *et al.*, 1999. As our data mainly focused on primary VMPC, which are a developmental cell type and Lu *et al.* examined normal adult and tumour primary cell cultures, the difference in results might reflect the different stages of differentiation between developmental and adult normal and cancer cell types. Androgens may regulate *Fgf10* gene expression in adult and tumour cultured cells, but do not affect *Fgf10* transcripts expressed during development. *Fgf10* transcripts are also expressed in the female VMP (i.e. in the absence of testosterone), as well as in the lung, limb buds, pancreas, kidneys, breast and skin (Bellusci *et al.*, 1997b; Bhushan *et al.*, 2001; Mailleux *et al.*, 2002; Ohuchi *et al.*, 2000; Ohuchi *et al.*, 1997; Tao *et al.*, 2002; Thomson and Cunha, 1999). Taken together it is evident that *Fgf10* is not directly androgen-regulated during prostate development even though prostate development is androgen-dependent.

TGFbeta has been shown to influence prostate development (Itoh *et al.*, 1998a), and probably mediates stromal-epithelial interactions (Blanchere *et al.*, 2001; Timme *et al.*, 1994). Previously the three isoforms of TGFbeta were shown to be present in normal prostate, BPH and prostate cancer (Danielpour, 1996; Knabbe *et al.*, 1993; Timme *et al.*, 1995). Beer *et al.*, 1997, observed a strong repression of *Fgf10* transcript levels in 3T3

Fgf10 transcript levels increased after 8 hrs of TGFbeta1 treatment. Hence it was decided to investigate if TGFbeta1 regulates *Fgf10* transcript levels in primary VMPC. Here, TGFbeta1 repressed *Fgf10* transcripts levels in both NIH3T3 cells and primary VMPC. It was demonstrated that all three cell-types expressed *Tgfbeta receptor type I* cultured in the presence of TGFbeta1 for 48 hrs. However only primary VMPC did not express *Tgfbeta receptor type I* in the absence of TGFbeta1. Firstly this suggests that TGFbeta1 regulates its' receptor expression and this has been previously observed (Kyprianou and Isaacs, 1988; Wikstrom et al., 1999). Also this raises the question of whether TGFbeta1 induces receptor expression rapidly enough to mediate TGFbeta1 action. Repression of *Fgf10* transcript levels was observed after three hours suggesting that the receptor must have been expressed. In retrospect, *Tgfbeta receptor type I* expression should have been examined at all time points. NIH3T3 *Fgf10* transcript levels were repressed after 1 hr and remained at low levels throughout culture. *Fgf10* transcript levels were also repressed in primary VMPC cultured with TGFbeta1, but transcript levels recovered after 48 hrs. *Fgf10* transcript levels were not decreased in PS-1 cells cultured for 48 hrs with TGFbeta1. As these cells expressed relatively low levels of *Fgf10* transcripts and TGFbeta1 represses *Fgf10* transcript levels, PS-1 cells were not used in any more TGFbeta1 treatments. Hence the rapid effects of TGFbeta1 on *Fgf10* transcript levels were not followed up in PS-1 cells. Overall these observations suggest that TGFbeta1 negatively regulates *Fgf10* transcript levels in primary prostate cells.

In the prostate, androgens negatively regulate the expression of TGFbeta ligands (Itoh et al., 1998a; Kyprianou and Isaacs, 1988; Wikstrom et al., 1999). After reduction of androgens by castration, the expression of TGFbeta1 in the prostate increases and can be suppressed by *in vivo* treatment with androgens (Bacher et al., 1993; Itoh et al., 1998a; Kyprianou and Isaacs, 1989). More recently it has been shown that ligand-bound AR inhibits TGFbeta transcriptional responses through selectively repressing the binding of Smad3 to the Smad binding element (Chipuk et al., 2002). In primary VMPC a TGFbeta1-mediated transcriptional response of *Fgf10* transcription has been identified,

and an experiment was designed to determine if testosterone inhibits this response. *Fgf10* transcript levels in primary VMPC grown in the presence of testosterone and TGFbeta1 were not repressed. This demonstrated that in our system testosterone was able to inhibit TGFbeta1 action over seven hours.

In conclusion, in primary VMPC testosterone did not regulate *Fgf10* transcript levels. TGFbeta1 repressed *Fgf10* transcript levels, and this transcriptional response to TGFbeta1 was inhibited with testosterone. This has identified a complex network of interactions in regulating *Fgf10* transcript levels in isolated prostate cells.

5 Regulation of *Fgf10* Transcript Levels in Organ Rudiments

5.1 Introduction

This chapter focuses on the regulation of *Fgf10* gene expression in prostatic organ rudiments by testosterone and TGFbeta1. Previous studies have shown that *Tgfbeta* isoforms are expressed in the prostate (Timme et al., 1995) but none have thoroughly addressed their transcript ontogeny during development. *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* transcript levels in the male UGT and VP during development were analysed by RNase protection assay. In chapter 4, it was shown that *Fgf10* gene expression in primary VMPC was not regulated by testosterone but was regulated by TGFbeta1, so the objective of this chapter was to see if regulation of *Fgf10* by TGFbeta1 in primary cells could be replicated in organ rudiments such as the VMP and VP. Male and female reproductive tracts were cultured under serum-free conditions in the presence or absence of testosterone and/or TGFbeta1 to address if these factors regulated *Fgf10* gene expression in prostatic rudiments.

5.2 Results

5.2.1 *Tgfbeta1*, -2 and -3 Transcript Levels in Male UGT and VP

The temporal expression pattern of *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcripts was examined in the male UGT and VP during development, by RNase protection assay (Fig. 5.1). In the male UGT, *Tgfbeta1* and *Tgfbeta3* transcript levels were low at e17.5, increased to maximum levels at P2, and were expressed at low levels at P6 (Fig. 5.1A). *Tgfbeta2* transcript levels decreased from e17.5 to e19.5, but increased to maximum

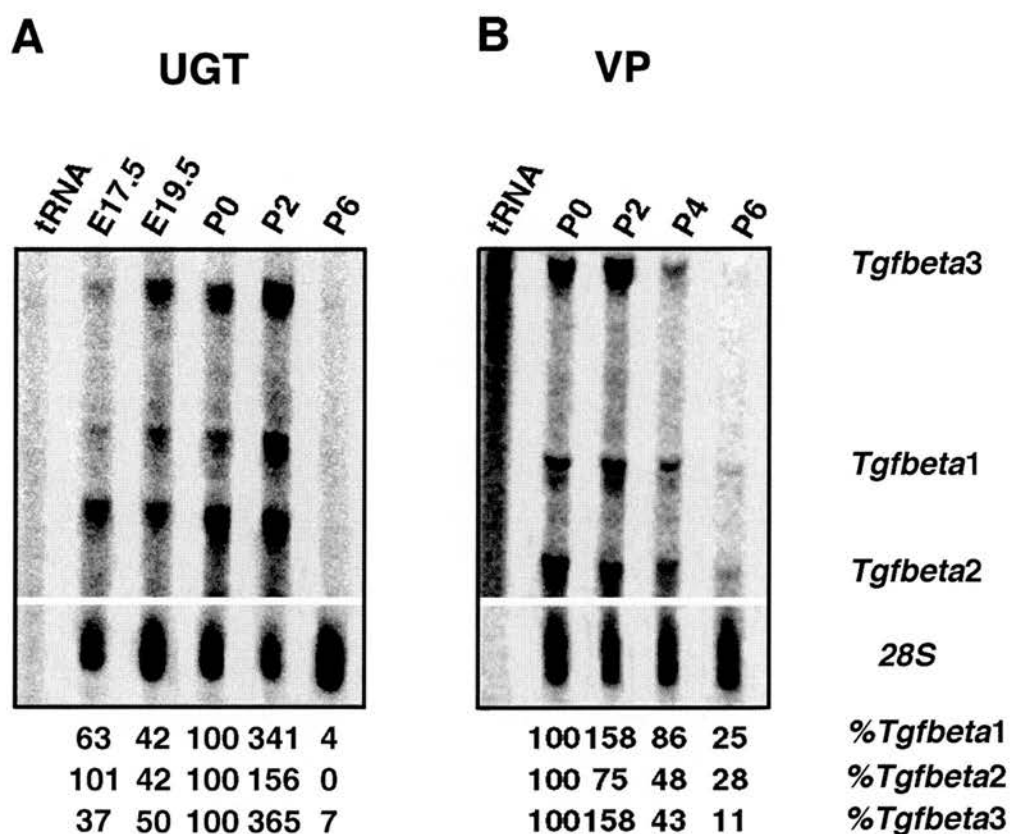


Fig. 5.1 Analysis of *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcripts in the male rat UGT and VP, by RNase protection assay. RNA was hybridised with ³²P-labelled antisense riboprobes for *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* and 28S as an internal standard. Transcript levels were quantified using a phosphorimager and normalised to 28S. Changes in transcript levels were calculated as percentages of P0 UGT for panel A and P0 VP for panel B. *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcript levels were highest in P2 UGT and were almost absent by P6 UGT (panel A). *Tgfbeta1* and 3 transcripts were highest in P2 VP and *Tgfbeta2* transcripts highest at P0 VP (panel B). In P6 UGT and P6 VP *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcripts were expressed at low levels.

levels at P2, and decreased again to low levels at P6. Analysis of transcript levels in embryonic VPs was not practical before P0 owing to difficulties in precise microdissection of VPs. Also embryonic VPs are small and as relatively large numbers of VPs would have to be collected to get enough RNA for an RNase protection assay, harvesting embryonic VPs was decided to be impractical. In the postnatal VP, *Tgfbeta* transcripts decreased with increasing age (Fig. 5.1B). Maximum transcript levels were observed at P0 for *Tgfbeta2* and P2 for *Tgfbeta1* and *Tgfbeta3*. All transcript levels had decreased between 4- and 10-fold by P6.

5.2.2 Regulation of *Fgf10* Transcript Levels in the VMP and UR

As TGFbeta1 repressed *Fgf10* transcript levels in primary VMPC, experiments were designed to determine if the same effect would be observed in female organ rudiments. Serum-free *in vitro* organ cultures were performed to investigate if TGFbeta1 and/or testosterone regulated *Fgf10* transcript levels in the VMP or UR. Female UGSs were cultured for either three-days or seven-hours.

5.2.2.1 3-Day Testosterone and/or TGFbeta1 Culture

Female P0 rat UGSs were initially cultured for three days to address the long-term regulation of *Fgf10* transcript levels in female organ rudiments by testosterone and/or TGFbeta1 (Fig. 5.2). These experiments were performed as a comparison to the long-term treatments carried out on primary VMPC. VMP+URs and URs were microdissected after culture, using the microdissection method shown in Fig. 5.2A, and their *Fgf10* transcript levels were analysed by RNase protection assay (Fig 5.2B.). All *Fgf10* transcript levels were standardised to untreated VMP+UR *Fgf10* transcript levels. *Fgf10* transcript levels were approximately 4-fold less in the UR than VMP+UR. *Fgf10* transcript levels were not affected in VMP+UR or UR by a 3-day TGFbeta1 treatment. This correlates with the regulation of *Fgf10* gene expression observed in primary VMPC, as a long-term TGFbeta1 treatment did not affect *Fgf10* transcript levels in

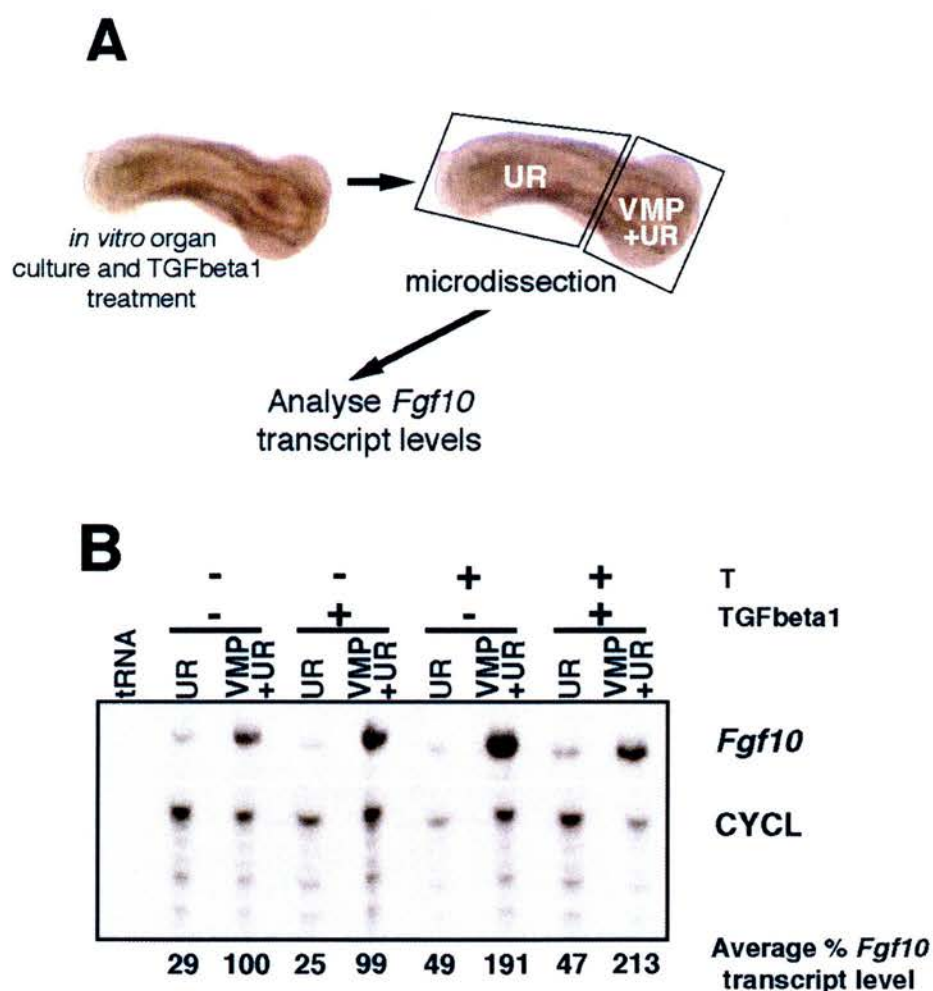


Fig. 5.2 Regulation of *Fgf10* transcript levels by TGFbeta1 and testosterone in the female UGS.

P0 female rat UGSs were cultured for three days in the presence or absence of testosterone (10^{-8} M) and/or TGFbeta1 (10ng/ml). VMP+UR and UR were microdissected after treatment (panel A) and RNA prepared. VMP+UR and UR *Fgf10* transcript levels were assayed by RNase protection (panel B), quantified using a phosphoimager and normalised to cyclophilin (CYCL). The numbers below panel B represent the average transcript abundance of *Fgf10* relative to levels in the VMP+UR cultured in the absence of TGFbeta1 ($n=2$). *Fgf10* transcript levels in VMP+UR and UR cultured with testosterone increased by two fold. Organ rudiments grown in the presence of TGFbeta1 resulted in no change in *Fgf10* transcript levels.

primary VMPC. However *Fgf10* transcript levels increased by two-fold in VMP+UR and UR when cultured for 3-days in the presence of testosterone, which did not correlate with regulation of *Fgf10* transcript levels in primary VMPC. However the effect observed in the female UGS was most likely not a direct regulation of *Fgf10* gene expression but was more likely to be due to an increase in the number of cells that express *Fgf10* transcripts; the VMP (the region of the female UGS that expresses the majority of *Fgf10* transcripts) appeared to be larger in UGSs cultured for three days with testosterone than those cultured with vehicle. Also UR stroma that expressed *Fgf10* transcripts may have also increased in size resulting in the two-fold increase of transcript levels, although this change was not as obvious as the change in the VMPs size.

5.2.2.2 7-Hour TGFbeta1 Culture

Next it was decided to address the short-term regulation of *Fgf10* transcript levels in female UGSs. Female P0 rat UGSs were cultured over-night in serum-free conditions, then cultured in the presence or absence of TGFbeta1 for seven hours (Fig. 5.3 and 5.4). Culturing organ rudiments with testosterone over seven hours was not performed, as testosterone had no effect on *Fgf10* transcript levels in primary VMPC and had no direct effect on *Fgf10* transcript expression in the female UGS. VMPs and URs were micro-dissected after culture in two different ways. Initially VMPs were micro-dissected as shown on Fig. 5.3A and 5.2A. As the micro-dissected VMP had UR attached (VMP+UR), VMP's were also micro-dissected as shown on Fig. 5.4A (VMP). *Fgf10* transcript levels in micro-dissected VMP+UR, VMP and UR were analysed using RNase protection assay (Fig. 5.3B and 5.4B).

A 1.7-fold decrease and a three-fold decrease in *Fgf10* transcript levels were observed in the VMP+UR (Fig. 5.3B.) and the VMP (Fig. 5.4B.), respectively, after treatment with TGFbeta1. No change was observed in *Fgf10* transcript levels in the UR. These results demonstrate that TGFbeta1 regulated *Fgf10* gene expression in the VMP but did not regulate *Fgf10* gene expression in the UR. This suggests that different mechanisms may

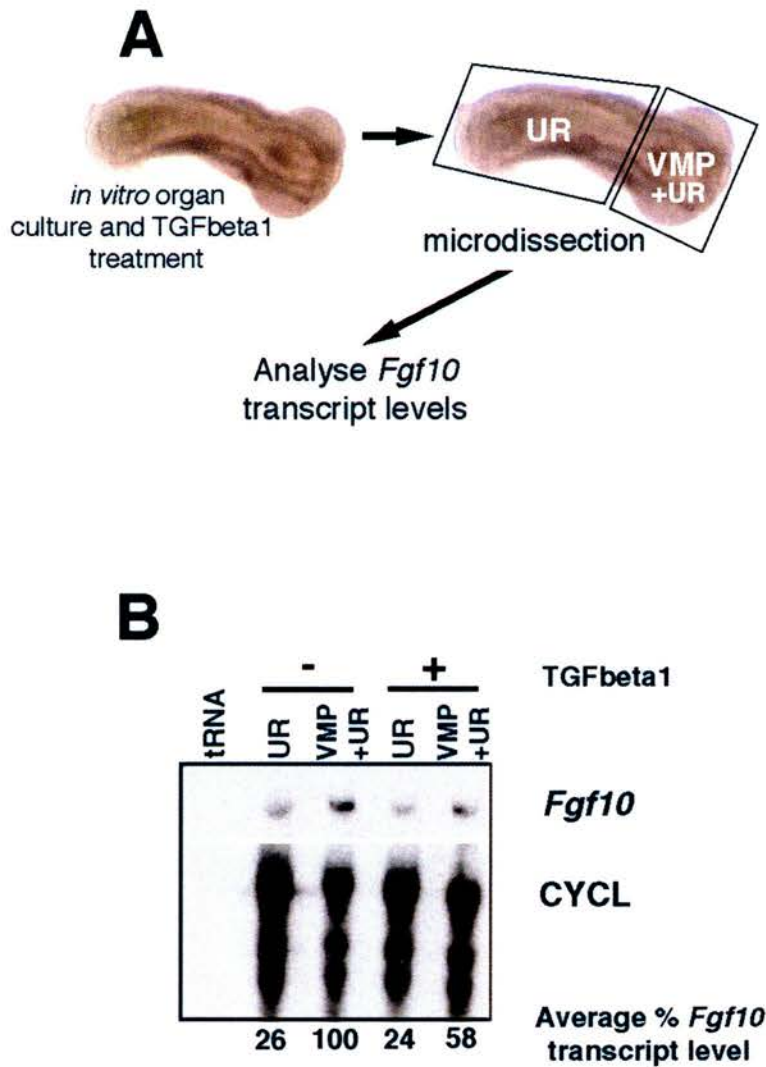


Fig. 5.3 Regulation of *Fgf10* transcript levels in the female UGS by TGFbeta1 for 7hrs.

P0 female rat UGS were culture overnight under serum free conditions then treated for 7hrs in the presence or absence of TGFbeta1 (10ng/ml) . VMP+UR and UR were microdissected after treatment (panel A); the VMP sample was microdissected with UR attached (VMP+UR). VMP+UR and UR *Fgf10* transcript levels were assayed by RNase protection (panel B), quantified using a phosphoimager and normalised to cyclophilin (CYCL). The numbers below panel B represent the average transcript abundance of *Fgf10* relative to levels in the VMP+UR cultured in the absence of TGFbeta1 (n=3). *Fgf10* transcript levels decreased by 1.7 fold in VMP+UR cultured with TGFbeta1. No change was a observed in *Fgf10* transcript levels in the UR.

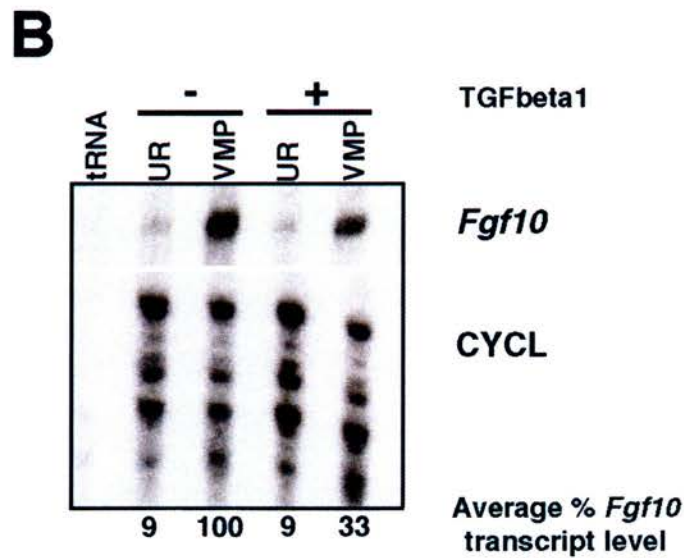
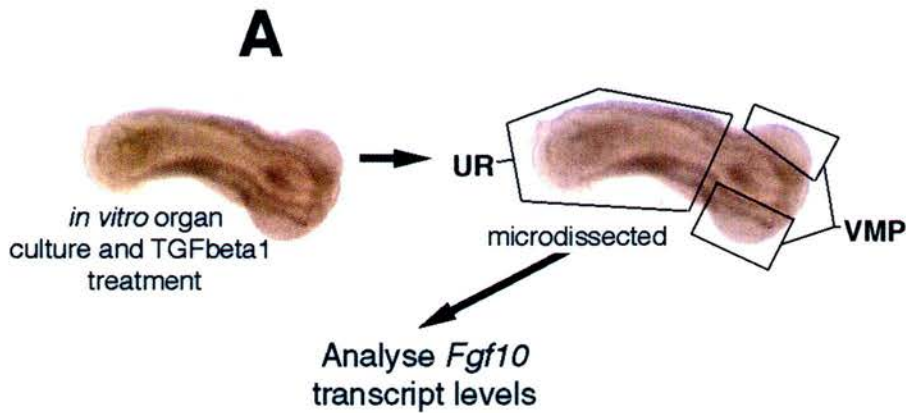


Fig 5.4 Regulation of *Fgf10* transcript levels in the female UGS by TGFbeta1 for 7hrs.

P0 female rat UGS were cultured overnight under serum free conditions then treated for 7hrs in the presence or absence of TGFbeta1 (10ng/ml). VMP and URs were microdissected after treatment (panel A); VMPs were microdissected so that no UR was attached. VMP and UR *Fgf10* transcript levels were assayed by RNase protection (panel B), quantified using a phosphorimager and normalised to cyclophilin (CYCL). The numbers below panel B represent the average transcript abundance of *Fgf10* relative to levels in the VMP cultured in the absence of TGFbeta1 (n=3). UGS cultured with TGFbeta1 resulted in a three fold decrease of *Fgf10* transcript levels in the VMP. No change was observed in *Fgf10* transcript levels in the UR.

control *Fgf10* gene expression in cells located in different parts of the UGS. However there is still the question of whether testosterone could block the three-fold down-regulation in VMP organs by TGFbeta1. This was not addressed owing to time limits on this thesis and that the experiments performed on primary VMPC were performed towards the end of these experiments.

5.2.3 *Fgf10* Transcript Levels in VPs Cultured with TGFbeta1 and/or Testosterone

It was shown that TGFbeta1 regulated *Fgf10* transcript levels both in primary VMPC and in VMP organ rudiments. Next the effect of TGFbeta1 and/or testosterone on *Fgf10* transcript levels was examined in VPs.

5.2.3.1 6-Day Testosterone and TGFbeta1 Culture

Initially, P0 VPs were cultured for six-days in the presence and absence of testosterone and/or TGFbeta1. Whole mount images of VPs, after a six-day culture in the presence and/or absence of testosterone and/or TGFbeta1, are shown on Fig. 5.5A. A six-day TGFbeta1 treatment in the absence of testosterone caused a small decrease in *Fgf10* transcript levels (Fig. 5.5B.). VPs cultured in the presence of testosterone and TGFbeta1 showed no significant change in *Fgf10* transcript levels compared to VPs cultured only in the presence of testosterone. Only small changes were observed in *Fgf10* transcript levels over this period of time. A noticeable difference in the phenotype of VP architecture cultured with TGFbeta1 suggested that differences in epithelial to mesenchymal ratios may be masking the effect of TGFbeta1 on *Fgf10* transcript levels (i.e. if the addition of TGFbeta1 caused a reduction in the epithelial content then the ratio of cells that express *Fgf10* to those that do not would increase. This would indirectly cause an increase in *Fgf10* transcript levels observed by RNase protection assay). However it was reported that very little change (1.3-fold increase) in the

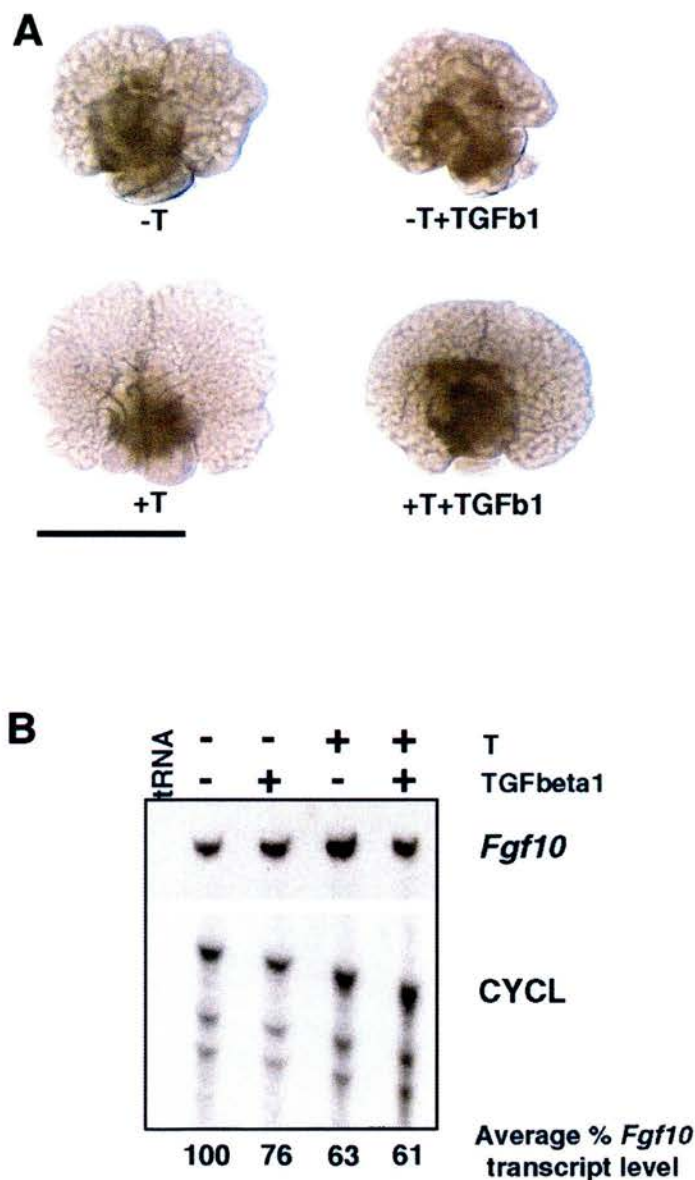


Fig. 5.5 Regulation of *Fgf10* transcript levels by TGFb1 in VPs cultured for 6 days. P0 male rat VPs were cultured for six days in the presence and absence of testosterone (10^{-8} M) and/or TGFb1 (10ng/ml) (Panel A, scale bar = 1mm). VP *Fgf10* transcript levels were assayed by RNase protection, quantified using a phosphorimager and normalised to cyclophilin (CYCL). The numbers below panel B represent the average transcript abundance of *Fgf10* relative to levels in VPs cultured in the absence of testosterone and TGFb1 (n=3). Panel B, six day TGFb1 treatment in the absence of testosterone caused a small decrease in *Fgf10* transcript levels. VPs cultured in the presence of testosterone and TGFb1 showed no significant change in *Fgf10* transcript levels compared to VPs cultured in the presence of testosterone.

epithelial to mesenchyme ratio was observed in VPs cultured for 6-days in the presence of testosterone to that in VPs cultured in the absence of testosterone using CK19 as an epithelial marker (Thomson et al., 1997). Hence a direct comparison between VPs cultured with and without testosterone can be made. The results presented here suggest that testosterone caused a small decrease in *Fgf10* transcript levels over six days. More recent unpublished data suggests that there is a change in mesenchyme to epithelial ratio by testosterone using vimentin as a mesenchymal marker (C. Grace and A.A. Thomson, unpublished). This raises a question of whether there was a change in ratio of cell types. To overcome this problem VPs were cultured for less time in the presence of testosterone so less epithelial growth and mesenchymal differentiation would have occurred.

5.2.3.2 3-Day Culture with Testosterone followed by a Seven Hour TGFbeta1 Treatment

On day three of culture, VPs were cultured in the presence and absence of TGFbeta1 for seven hours. This was performed as the VPs were larger than those grown for less time so more RNA could be obtained from fewer organs, and by only culturing VPs for seven hours with TGFbeta1 relatively little structural differences were observed between different treatment groups. This meant any large changes in *Fgf10* transcript levels were due to direct regulation on the *Fgf10* gene and not due to changes in cell ratios. After three days in culture, although obvious differences in epithelial branching are apparent in organs cultured with and without testosterone, VPs visually appeared more similar in epithelial and mesenchymal ratios than those cultured for six days (Fig. 5.6A). A seven-hour TGFbeta1 treatment caused a small decrease in *Fgf10* transcript levels both in the presence and absence of testosterone (Fig. 5.6B).

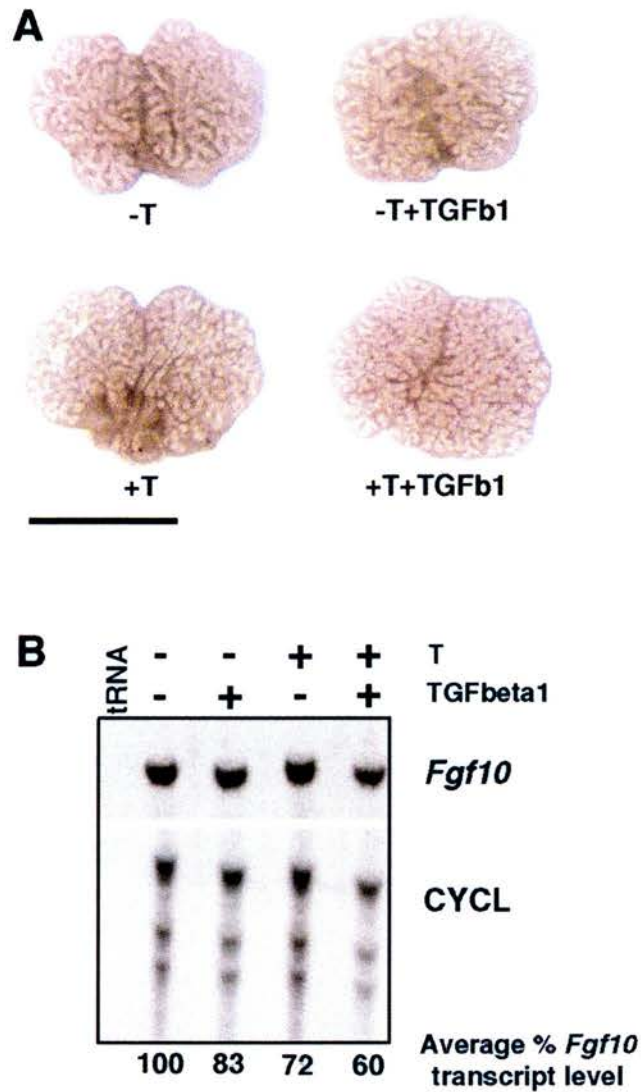


Fig 5.6 Regulation of *Fgf10* transcript levels by TGFbeta1 in VPs cultured for three days.

P0 male rat VPs were cultured for three days in the presence and absence of testosterone (10^{-8} M) then treated for seven hours (panel A) in the presence and absence of testosterone and/or TGFbeta1 (10ng/ml) (scale bar = 1mm). VP *Fgf10* transcript levels were assayed by RNase protection, quantified using a phosphoimager and normalised to cyclophilin (CYCL). The numbers below panel B represent the average transcript abundance of *Fgf10* relative to levels in VPs cultured in the absence of testosterone and TGFbeta1 (n=2). 7hr TGFbeta1 treatment caused a small decrease in *Fgf10* transcript levels in the presence and absence of testosterone.

5.2.3.3 Three- and Seven-hour TGFbeta1 Culture

The fold-change of *Fgf10* transcript levels in VPs cultured with TGFbeta1 for seven hours was not as large as that observed in primary VMPC and VMP rudiments. There are several reasons that may account for this. But it may be an effect of factors produced in the epithelium that also regulated *Fgf10* gene expression. In VPs cultured overnight there appeared to be less epithelial branching than VPs grown for longer periods of time (Fig. 5.7A and B compared to Fig. 5.5A and Fig. 5.5B). Again culturing organ rudiments with testosterone over seven hours was not performed, as testosterone had no effect on *Fgf10* transcript levels in primary VMPC and had no direct effect on *Fgf10* transcript expression in the female UGS.

Fgf10 transcripts levels (Fig. 5.7C) decreased by 1.5-fold in VPs cultured in the presence of TGFbeta1 for 3hrs (Fig. 5.7A). *Fgf10* transcript levels decreased by 1.3-fold (Fig. 5.7D) in VPs cultured in the presence of TGFbeta1 for 7hrs (Fig. 5.7B). These fold-changes were not equivalent to those observed in primary VMPC and VMP rudiments cultured with TGFbeta1, but were larger than those seen in VPs with more epithelium. This suggests epithelium might be inhibiting the effect of TGFbeta1 on mesenchymal cells in VPs.

5.3 Summary of Results

Experiments were performed to determine whether *Tgfbeta* isoforms were expressed during prostatic development, and if TGFbeta1 and/or testosterone regulated *Fgf10* gene expression in organ rudiments. Previous studies have shown that *Tgfbeta* isoforms were present in normal prostate, BPH, and prostatic cancer (Danielpour, 1996; Knabbe et al., 1993; Timme et al., 1995), yet few studies have thoroughly examined their expression

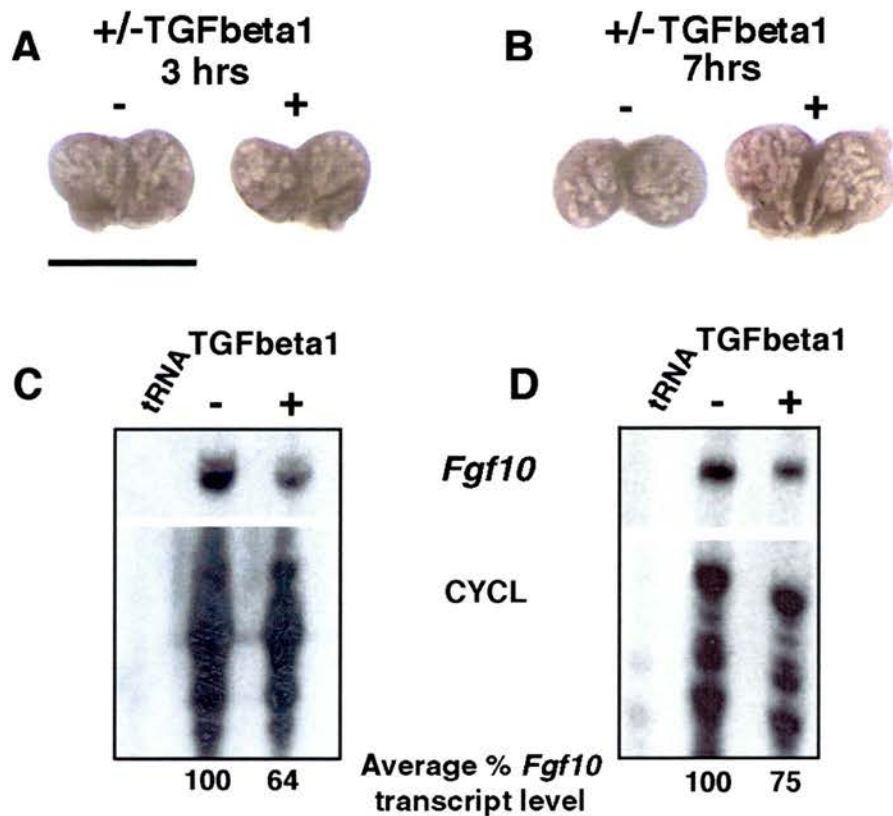


Fig. 5.7 Regulation of *Fgf10* transcript levels in VPs by TGFbeta1.

P0 male rat VPs were cultured and treated for three (panel A) or seven hours (panel B) in the presence (+) or absence (-) of TGFbeta1 (10ng/ml) (scale bar = 1mm). VP *Fgf10* transcript levels were assayed by RNase protection, quantified using a phosphoimager and normalised to cyclophilin (CYCL). The numbers below panel C and D represent the average transcript abundance of *Fgf10* relative to levels in VPs cultured in the absence of TGFbeta1 (n=2). VPs cultured in the presence of TGFbeta1 for 3hrs result in a 1.5 fold decrease in *Fgf10* transcript levels (panel C). VPs cultured in the presence of TGFbeta1 for 7hrs result in a 1.3 fold decrease in *Fgf10* transcript levels (panel D).

during embryonic and neonatal prostate development. *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcript levels were analysed in male UGTs and VPs during development of the prostate. *Tgfbeta1* and *Tgfbeta3* transcript levels were low in the male UGT around the start of prostate development (e17.5) and increased up to P2. Low levels of *Tgfbeta1* and *Tgfbeta3* transcripts were detected at P6. *Tgfbeta2* transcript levels in the male UGT were relatively high around the start of prostate development, were lower at e19.5 and elevated at P2. Lower levels of transcripts were detected at P6. In the VP, *Tgfbeta1* and *Tgfbeta3* transcript levels peaked at P2 and decreased to P6. *Tgfbeta2* transcript levels were highest at P0 and again decreased to P6. The observed changes in *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcript levels correlated with the growth pattern of the VP. VP growth and branching morphogenesis occurs in the early neonatal period, becoming quiescent after P20. This suggested that TGFbeta-signalling may participate in early VP development, which agrees with previous results that suggested *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* isoforms are associated with fetal UGS tissues and therefore prostatic morphogenesis (Timme et al., 1994). However, another report suggested *Tgfbeta1* expression remains constant and *Tgfbeta2* and *Tgfbeta3* are inversely related during prostatic development (Itoh et al., 1998a). This report examined *Tgfbeta* expression patterns at P1, P20, P60 and P100 and compared values to P20. Our studies examined levels between e17.5 and P6, when the prostatic growth rate is at its highest, therefore providing a better picture of how *Tgfbeta* isoform expression changes with early prostate organogenesis.

Androgens are postulated to regulate *Fgf10* gene expression in the prostate (Lu et al., 1999). In this chapter it was observed that androgens caused a two-fold increase in *Fgf10* transcript levels both in the VMP+UR and UR cultured for three days. The increase in *Fgf10* transcript levels may be a result of androgens either stimulating proliferation of *Fgf10* expressing cells or promoting URE-mesenchymal interactions resulting in increased *Fgf10* gene expression (discussed in chapter 8). This would explain the increase in *Fgf10* transcripts levels in both the VMP+UR and UR and suggests that testosterone does not directly regulate *Fgf10* transcript levels. The

experiments performed on primary VMPC (chapter 4) and experiments performed on prostatic rudiments agree with this hypothesis (Thomson and Cunha, 1999). *Fgf10* transcript levels were not regulated by androgens in primary VMPC, and while *Fgf10* transcript levels increased after treatment with testosterone in the SV (and to a lesser degree in the VP), these changes were not sensitive to anti-androgen treatment (Thomson and Cunha, 1999). Thus *Fgf10* gene expression was unlikely to be directly regulated by androgens.

It has previously been shown that TGFbeta1 repressed *Fgf10* transcript levels in a 3T3 fibroblast cell type (Beer et al., 1997) and in lung mesenchyme cells and lung explants (Lebeche et al., 1999). We have observed a similar regulation by TGFbeta1 on *Fgf10* transcript levels in VMP organ rudiments but not in UR rudiments. *Fgf10* transcript levels were decreased by a maximum of three-fold in the VMP when cultured for seven hours with TGFbeta1, similar to changes observed in primary VMPC. Also as in primary VMPC *Fgf10* transcript levels were not regulated by a longer exposure to TGFbeta1 in the VMP. This suggests that the effect of TGFbeta1 was reversible in organ rudiments similar to what was observed in primary VMPC. TGFbeta1 did not affect *Fgf10* transcript levels in the UR under any treatment regimes. This suggested that *Fgf10* gene expression was regulated by different mechanisms in different parts of the female UGS or that regulatory signalling from the URE was preventing TGFbeta1-mediated repression of *Fgf10* transcript levels. Also this suggests that TGFbeta1 regulation of *Fgf10* transcript levels is VMP/VMPC specific.

To determine if TGFbeta1 or testosterone might regulate *Fgf10* gene expression in the VP, they were added together to organ cultures of VPs. Over a six-day culture, TGFbeta1 did not appear to regulate *Fgf10* transcript levels in VPs in the presence of testosterone, but a small decrease in *Fgf10* transcript levels was observed in the absence of testosterone. *Fgf10* transcript levels were also decreased in VPs cultured with testosterone alone, which agrees with published data that suggested testosterone does not directly up-regulate *Fgf10* gene expression in the VP (Thomson and Cunha, 1999).

A problem resulting from culturing for six days with hormones or growth factors when examining a factor only expressed in the mesenchyme, is differing final ratios of mesenchyme to epithelium. No experiments have been performed to address if TGFbeta1 changes the ratio of cell types in VPs culture over three and six-days. However the effect of testosterone on mesenchymal to epithelial ratios has been examined. It was published that VPs cultured with and without testosterone have similar levels of CK19 (only a 1.3 fold increase in organs cultured with testosterone) (Thomson et al., 1997), suggesting that there was very little change in epithelial to mesenchymal ratio. However recently it was demonstrated that VPs cultured in the absence of androgens have a higher level of vimentin expression (O.C. Grace and A.A. Thomson, unpublished); and as vimentin stains mesenchymal cells, VPs cultured in the absence of testosterone may contain more cells that express *Fgf10* transcripts. These results may differ as testosterone may regulate the expression of either vimentin or CK19, or cause differentiation of cells and thus possibly alter their expression. To try and resolve these problems VPs were cultured for shorter periods of time with testosterone, and then treated with TGFbeta1 for seven hours (i.e. the period of time that TGFbeta1 was shown to affect *Fgf10* transcript levels in primary VMPC and VMP rudiments). This ensured that major differences between the ratio of mesenchymal to epithelial cells were not affecting *Fgf10* transcript levels. After a three-day serum-free culture in the presence or absence of androgens, the VPs appeared to be of comparable size and therefore they would more likely have similar mesenchymal to epithelial ratios. Again *Fgf10* transcript levels were not increased in VPs cultured with testosterone and it was observed that TGFbeta1 caused only a small decrease in *Fgf10* transcript levels both in the presence and absence of testosterone. This suggests that testosterone did not antagonise the regulation of *Fgf10* transcript levels by TGFbeta1.

It was postulated that the effect of TGFbeta1 on *Fgf10* transcripts levels was inhibited by factors produced in the epithelium in the prostate, as factors produced in the epithelium have been proposed to regulate *Fgf10* gene expression during lung

development (Bellusci et al., 1997b). To test this hypothesis it was decided to treat VPs with TGFbeta1 on day one in culture (i.e. when epithelia content appeared to be at its lowest in our culture system). *Fgf10* transcript levels were consistently repressed over three- and seven-hours in culture with TGFbeta1. The degree of repression was not as great as what was seen in primary VMPC or the VMP rudiment. Hence in P0 VPs, TGFbeta1 had a greater effect on *Fgf10* transcript levels than in more developed VPs, because branching morphogenesis of the epithelium had relatively only just commenced.

In conclusion it was demonstrated that testosterone did not directly regulate *Fgf10* transcript levels in VP rudiments *in vitro*, but can indirectly alter levels of *Fgf10* transcripts in the female UGS by causing a proliferation of mesenchymal cells, and hence increasing the ratio of *Fgf10* expressing cells to non-expressing cells. TGFbeta1 can repress *Fgf10* transcript levels in male and female prostatic rudiments, however the level of repression is less in the VP compared to the VMP. The major difference between the VMP and VP is the epithelial content and it is possible that the epithelium counteracts the effect of TGFbeta1 on *Fgf10* transcript levels.

6. Analysis of the *Fgf10* Promoter

6.1 Introduction

To understand the mechanisms responsible for regulating *Fgf10* gene expression, analysis of the *Fgf10* promoter was performed. Sequences lying 5' to the *Fgf10* translation start site from mouse, human and sheep were compared using VISTA analysis to identify a region that may contain the *Fgf10* transcription start site. A fall in DNA sequence homology potentially indicated the presence of a transcription start site. An *Fgf10* transcription start site was mapped by RNase protection analysis using antisense riboprobes designed against the region of promoter identified by VISTA analysis. VISTA analysis was also used to identify regions of high sequence homology between mouse and human genomic sequence, 5' to the transcription start site. Conserved elements of the moused sequence between mouse and human genomic sequence were placed upstream of pGL3 SV40 promoter to test for enhancer activity. Using this method two regions were identified, one repressed and the other enhanced transcription. A deletion analysis of the *Fgf10* promoter was performed to analyse the activity of the conserved regions in the context of the native *Fgf10* core promoter. A 244 bp DNA fragment, that when deleted reduced *Fgf10* transcription, was examined for transcription factor binding sites. An Sp1 site was identified in this region, and was shown to promote *Fgf10* transcription, and mediate repression of *Fgf10* expression via TGFbeta1.

This project was facilitated by the gift of a 6.6 kb *Fgf10* genomic clone of mouse DNA isolated by Dr. Justin Grindley. Dr. Grindley also supplied mouse and human *Fgf10* genomic sequences used in the VISTA analysis, and cloned the mouse regions, identified as being homologous to human, into the pGL3 SV40 vector. The work described here was performed in prostate cells in collaboration with Dr. Grindley, and compliments his studies in lung cells.

6.2 Results

6.2.1 Identification of an *Fgf10* Transcription Start Site

To identify regions of a mouse *Fgf10* genomic clone that might contain a transcription start site, a comparison of mouse and human *Fgf10* genomic sequences and sheep cDNA sequence (GenBank acc no. AF213396) was performed using VISTA analysis (Fig. 6.1). VISTA is a program for visualizing global DNA sequence alignments of arbitrary length (Mayor et al., 2000), and the program determines the identity between two DNA sequences and displays this as a graph. For these studies high sequence conservation was defined as 50 % or greater. The comparison between mouse and human genomic sequence, and mouse genomic sequence and sheep cDNA sequence revealed a sharp dip in homology between -1300 bps and -1500 bps (relative to the translation start site) (Fig. 6.1). The dip in homology coincided with the 5' end of the sheep cDNA sequence and was therefore thought to represent a potential transcription start site, assuming that the cDNA was full length. Riboprobes were designed that flanked this region in the mouse to determine whether it contained an *Fgf10* transcription start site, and to map the start site position.

To map the transcription start site RNase protection was used with antisense riboprobes from six *Fgf10* DNA templates (Fig. 6.2A). DNA templates were synthesised either by restriction digestion of the *Fgf10* genomic construct or by PCR using primers complementary to *Fgf10* (see table 2.2), which were cloned into pGEM T-easy or pBluescript. Probe1 corresponded to a 457-bp *NheI/PstI* fragment 5' to the putative start site region. Probe2 corresponded to a 488-bp *PstI* fragment. Probes 3-6 were cloned by PCR using primers complementary to the *Fgf10* construct (Table 2.2). Labelled probes were hybridised with 10 µg of total RNA isolated from NIH3T3 cells, e13.5 mouse lung or P0 mouse VPs (Fig. 6.2B).

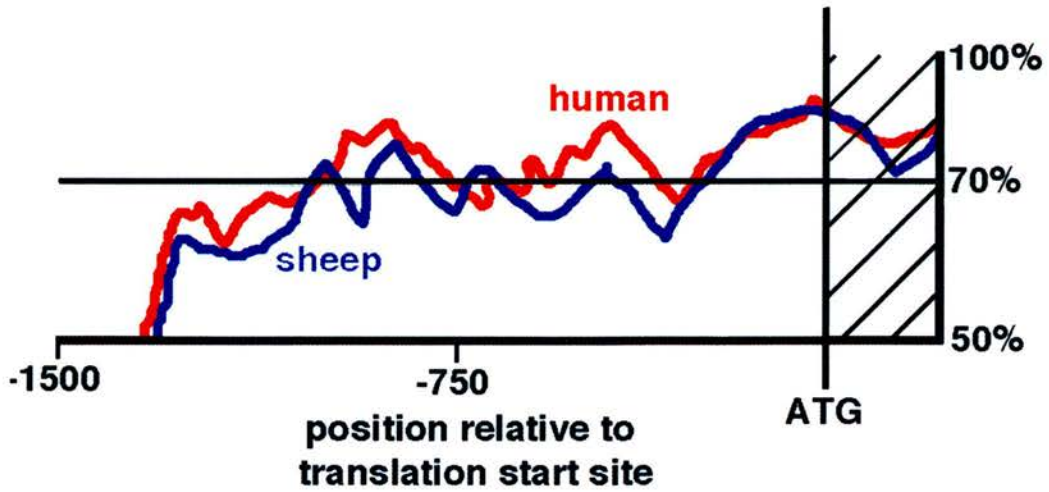


Fig. 6.1 Comparison of *Fgf10* sequences from three species to identify regions of homology and potential transcription start sites.

The sheep sequence was obtained from an NCBI blast search on the mouse *Fgf10* genomic sequence, using a non-redundant database. Comparison between species was performed using VISTA analysis and plotted as percentage homology compared to mouse. Comparison between mouse and human (red line), and mouse and sheep (blue line) revealed a sharp dip in homology between -1300bps and -1500bps (when ATG is -1,+1). The dip in homology represented a potential transcription start site. Riboprobes were designed flanking this region to determine whether it contained an *Fgf10* transcription start site.

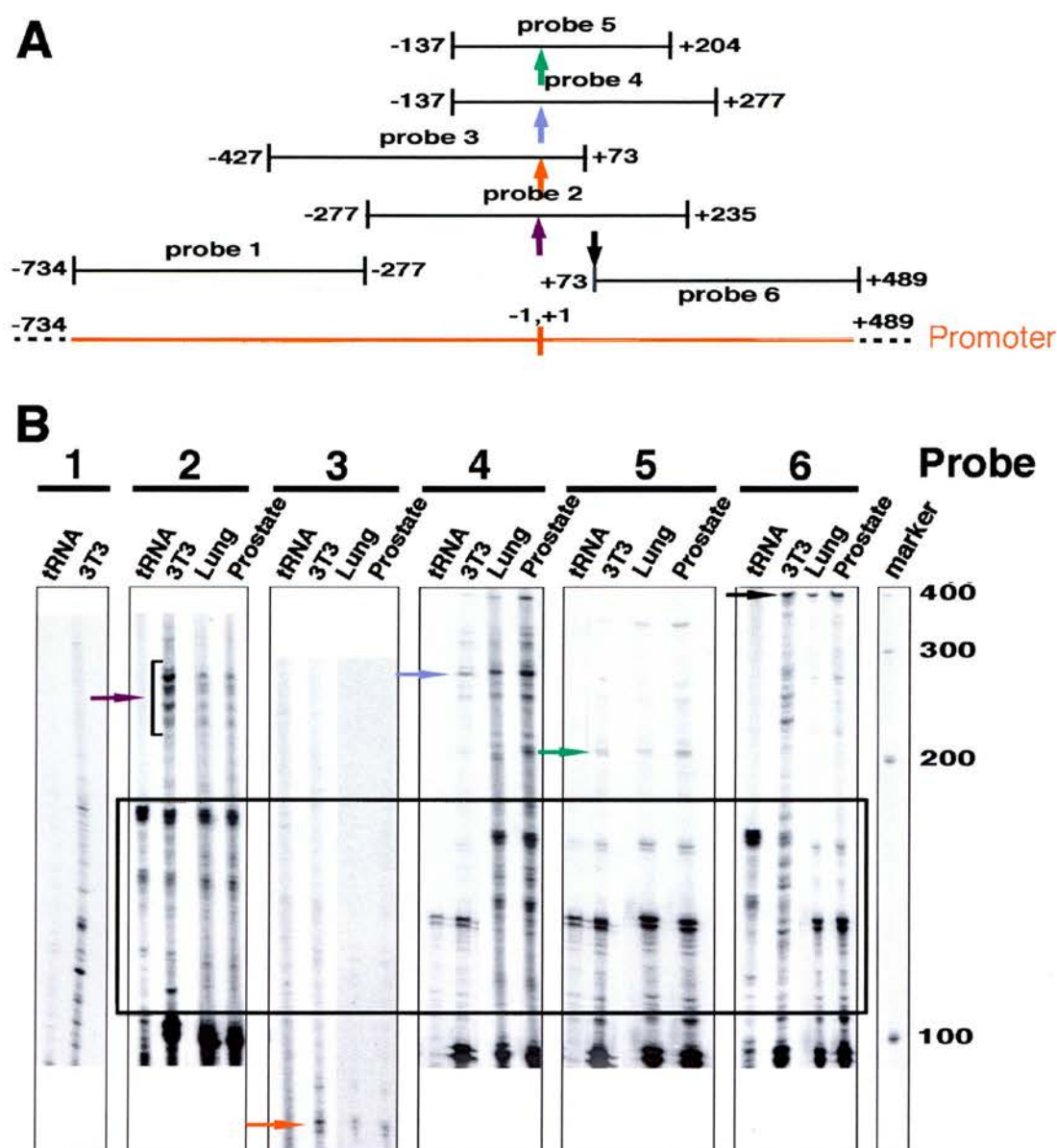


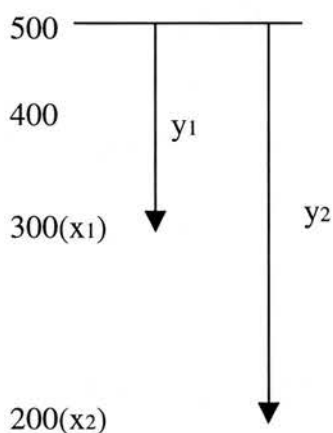
Fig. 6.2 Identification of an *Fgf10* transcription start site by RNase protection assay (RPA). Panel A. Position of riboprobes on *Fgf10* promoter region. Panel B. Riboprobes were prepared from genomic clones of the mouse *Fgf10* sequence. Mouse NIH-3T3, E13.5 lung and P0 VP RNA were hybridised with ^{32}P -labelled antisense riboprobes, shown in panel A. The figure consists of numerous RPAs exposed for different times and using different cyclophilin probes, which resulted in background internal protected bands. A boxed area, on panel B, surrounds the region where cyclophilin background bands occur. The marker lane is representative of the size of probes but each gel run had its own marker lane. Probe 1, numerous protected bands. Probe 2, numerous protected bands between 230nts and 270nts in length but no full length probe protection suggesting no up stream start sites. Probe 3, no full length probe present but protected band observed at about 70nts corresponding to protected bands in probe 2. Probe 4, main protected band at 277nts and probe 5 protected band at 204nts. Probe 6, full probe length protection shown. The riboprobes have elucidated a major *Fgf10* transcription start site 704nts 5' of the translation start site.

RNase-protected bands were observed for each riboprobe. The size of RNase-protected bands was used to calculate the putative transcription start site. To accurately measure the protected bands in nucleotides the following equation was used:

$$\log y = \log c + k \log x$$

where c = intercept and k = slope

An example of how the protected bands sizes were calculated is shown below.



As the majority of the protected bands fell between the size markers 200 to 300, this region was used to calculate the slope (k). The 500 nucleotide marker was taken to equal 0 and the 300 nucleotide (x_1) and 200 nucleotide markers (x_2) were measured in mm from this point, y_1 and y_2 respectively.

$$k = (\log y_1 - \log y_2) / (\log x_1 - \log x_2)$$

When the value of k had been calculated, the intercept (c) was calculated using the following equation:

$$\log c = k \log x_1 - \log y_1$$

The size of the protected bands was then accurately measured by measuring the distance (mm) from the 500 nucleotide marker and applying the equation:

$$\log y = \log c + k \log x$$

where y = distance in mm of protected bands from the 500 nucleotide marker, c = calculated intercept, k = calculated slope, and x = the size of the protected band to be calculated.

The position of the putative transcription start site was calculated by tracing the number of nucleotides of the protected bands from the 3' end of the probe on the *Fgf10* sequence. This was performed on each probe in each RNA sample, and the start site was identified from bands from four independent probes.

Using probe1, numerous protected bands were observed. Using probe2 numerous protected bands between 230 nts and 270 nts in length were detected but there was no full-length probe protection, suggesting that the start site lay within this region and that no start sites were present upstream of the 5' end of the probe. Probe3 was designed spanning probe1 and probe2 to address if the bands observed in these regions were putative start sites. Using probe3 a protected band of approximately 70 nts was observed corresponding to a protected band within the numerous bands observed using probe2. The observed protected band represented a putative transcription start site but it could not be accurately mapped to a single nucleotide. To map the exact position of the putative start site, probe4 and probe5 were designed. Probe4 gave a strong protected band at 277 nts, and probe 5 yielded 204 nts band. The bands observed in probe4 and probe5 mapped to the same nucleotide in the *Fgf10* sequence, strongly suggesting that this was a transcription start site. In contrast, the 416 bp probe6 was fully protected suggesting this probe lay within the transcribed region of the *Fgf10* gene. The same protection pattern for each probe was detected using NIH3T3 cell, mouse lung and

mouse VP RNA, demonstrating that this transcription start site for *Fgf10* is in the same position in NIH3T3 cells, in e13.5 lungs and in P0 VPs. The major *Fgf10* transcription start site (-1, +1; the strongest protected band) was calculated to be 704 nts 5' of the translation start site and this is shown on Fig. 6.3. However this band did not account for all the transcripts as the band intensity was not as strong as that observed in the fully protected probe, suggesting that may be other transcription start sites.

6.2.2 Homologous Regions of the Mouse and Human *Fgf10* Genomic Sequences

The objective of identifying homologous regions between the mouse and human genomic sequences was to identify sequences that regulate *Fgf10* transcription. Conserved segments were defined to be regions in which every contiguous subsegment of the human sequence was at least 70% identical to the mouse sequence. The comparable regions were named A to L on the two loci (Fig. 6.4). It was thought these conserved sequences might represent sequences that regulate *Fgf10* transcription. An EST (mouse cDNA clone, soares mammary gland; GenBank Acc AI552268) was found to be contained in region E so this region was thought unlikely to be involved in *Fgf10* regulation. All the homologous regions in the 5' UTR, except region E (an EST), were cloned into the pGL3 SV40.

6.2.3 Analysis of Conserved Regions by an Enhancer Assay

As regions of homology may contain sites that regulate *Fgf10* transcription the pGL3 SV40 constructs were co-transfected with a control vector that expressed β -galactosidase into NIH3T3 cells, primary URSC and primary VMPC (Fig. 6.5) to identify any putative enhancer or repressor regions. Also all the transfections were performed in media containing CPSR-1 (controlled process serum replacement-type 1), as CPSR-1 is a synthetic substitute for serum and does not contain cytokines or steroids that might

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ctacagtgga aaacaagtga acaangggct ccctgtaggg aagqgaatgg
ttcccqccqg aagqcacaaa attcggaaaq cacgcggaca actcgcgctg
gtggccacgc tqcnaacaca qqacgccqga gccqcaatta ncangactgc
angctgcqgg qcqctgcccc cacnqaagcg gcgaagaagg gaccgcagcc
                                     Sp1
cccagctcan aacaaaaggca tcgcgcactc ctctcgccca aanatcccc
→ gccccctccc cggtnccctc ccccnccctt ttctctggcg ttcccagcag
cttaagggttt canatgtccc accgccgttt gacccttcc cccctttntc
cacccttgca aatgaggttt gaccagcaga ggcagagccc acttttggtt
gaaaagcact gacatttaga ntccaggCTT CAACCTGTTT ACAAGCGGCT
      DPE →                                     +72
TTCCAAGGGA CTTGGAGGTG GAGAGAAGGG CCCAACAAAA CGCCAGCCGC
```

Fig. 6.3 Sequence of the mouse *Fgf10* promoter region

DNA sequence of 500 nucleotides from nucleotide -428 to +72 of the *Fgf10* promoter region. The putative Sp1 sites are marked in bold type. The Sp1 site examined in more detail later in the chapter is marked with an arrow. The CpG island is underlined from -391 to -117. The major transcriptional start site, identified by RNase protection, is marked with a blue arrow, and the initiator element (Inr) sequence is shown in red. The transcribed sequence is shown in capital letters and the bold capital letters represents the downstream promoter element (DPE).

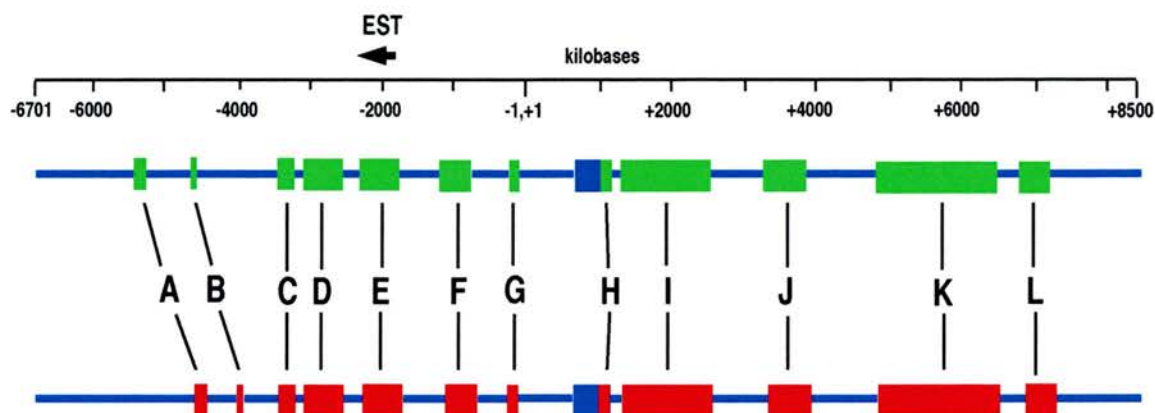


Fig 6.4 Comparison between mouse and human *Fgf10* promoter and intron sequences, Sequences were compared using VISTA analysis and represent a 70 % homology between sequences (originally performed by Justin Grindley). The diagram represents only 15.2 Kb of promoter and intronic *Fgf10* genomic sequence including only exon 1 (blue box) of the *Fgf10* gene. The comparable regions are labelled A to L on the two loci and are represented by boxes; green (top) and red (bottom) for mouse and human sequences, respectively. The blue line represents regions that show less than 70 % homology. Region E is an EST (mouse cDNA clone, soares mammary gland; GenBank Acc AI552268) located by a NCBI Blast search using an EST database. All the homologous regions, except region E, were cloned into the pGL3 SV40 promoter vector, by Justin Grindley, and analysed in enhancer transfection assays.

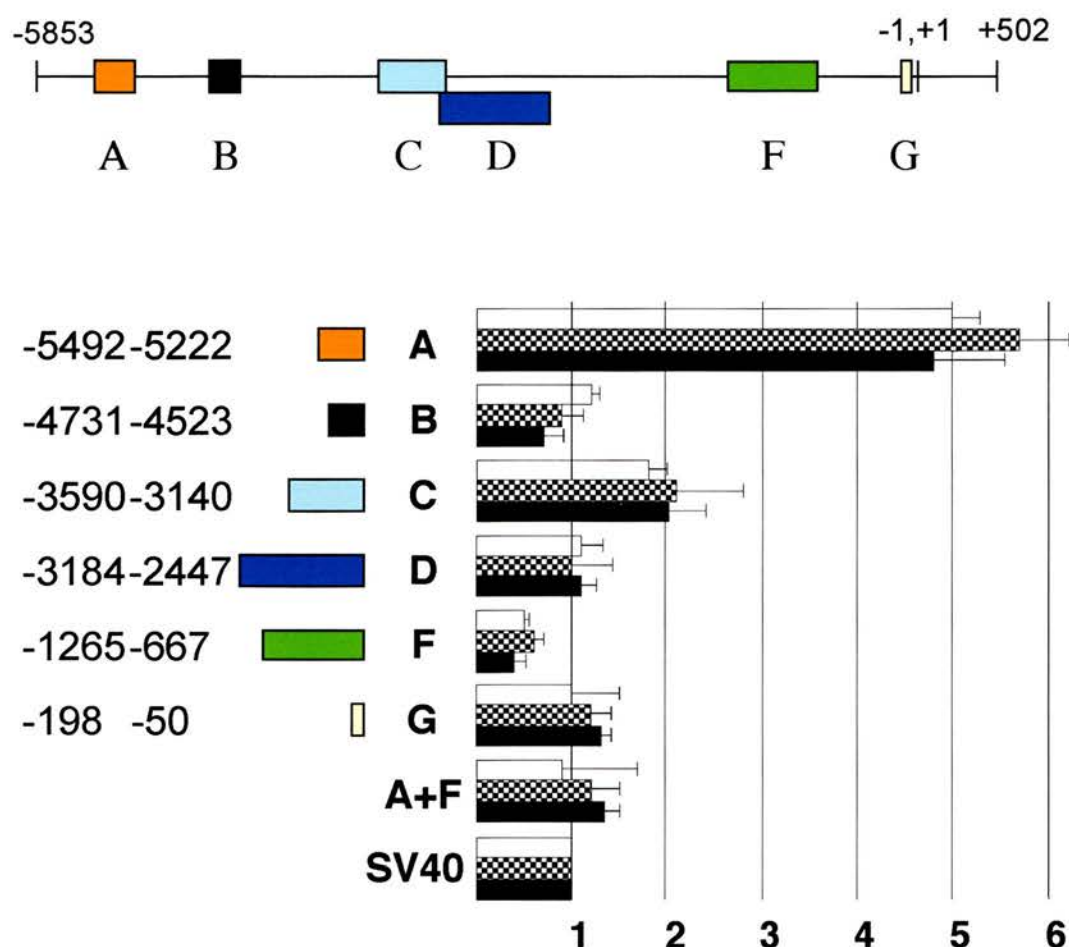


Fig. 6.5 Analysis of conserved regions using an enhancer assay.

Domains showing high DNA sequence conservation between the mouse and human *Fgf10* promoter region were cloned into SV40 pGL3 promoter vector. Constructs were co-transfected with a β gal expressing vector into NIH-3T3 (clear bars), URS (chequered bars) and primary VMPC (solid bars). Results shown are luciferase values normalised for transfection efficiency (by β gal assay) and standardised to SV40 luciferase activity. A, five-fold increase in activity in all three cell types. B, no change in activity. C, two fold increase in activity. D, no change in activity. F, two-fold down-regulation of activity. G, no change in activity. A+F (region F cloned 3' of A), no change in activity. Error bars represent SEM.

affect promoter activity. Luciferase values were normalised for transfection efficiency (by β -gal assay), and standardised to basal SV40 luciferase activity. Region A increased the luciferase activity of the SV40 vector five-fold, and region C increased luciferase activity two-fold, suggesting that these regions act as enhancer regions in the *Fgf10* promoter in all three cell types. Region F caused a two-fold decrease in luciferase activity suggesting that it is a repressor region. Regions B, D and G had no effect on the SV40 promoter vector. To determine if region F could repress activity caused by an enhancer region isolated from the *Fgf10* promoter, region F was cloned 3' of region A. Region F inhibited the activity induced by region A, suggesting that region F could repress *Fgf10* gene transcription induced by an enhancer. To determine if the regions identified by the SV40 enhancer assay affect the *Fgf10* core promoter in the same manner, a deletion analysis of the *Fgf10* promoter was performed.

6.2.4 Deletion Analysis of the *Fgf10* Promoter

6.6kb of mouse DNA 5' to the *Fgf10* translation start site, presumed to contain the *Fgf10* promoter, was cloned into the pGL3 basic vector (Promega) so that the coding region for *Fgf10* was replaced by a luciferase reporter gene fused at the translation start site. The plasmid pGL3 was found to have a high basal luciferase activity in our transfection system, and therefore the *Fgf10* promoter was cloned into pA3Luc. pA3Luc was originally constructed to prevent cryptic plasmid transcription, that can increase background activity (Wood et al., 1989). For our studies it was necessary to modify pA3Luc (pA3Lucm; section 2.2.6.2.1.) to aid the cloning of the *Fgf10* promoter regions (Section 2.2.6.2.). Constructs contained the regions of *Fgf10* promoter sequence are detailed in Fig. 6.6 and Table 2.4.

Promoter constructs were co-transfected with a β -gal expressing vector into NIH-3T3 and primary VMPC. Luciferase values were normalised for transfection efficiency (by β -gal assay) and standardised to the Full-length promoter luciferase activity (Fig. 6.6). In NIH-3T3 cells, 5' to 3' deletions resulted in a gradual increase in activity with

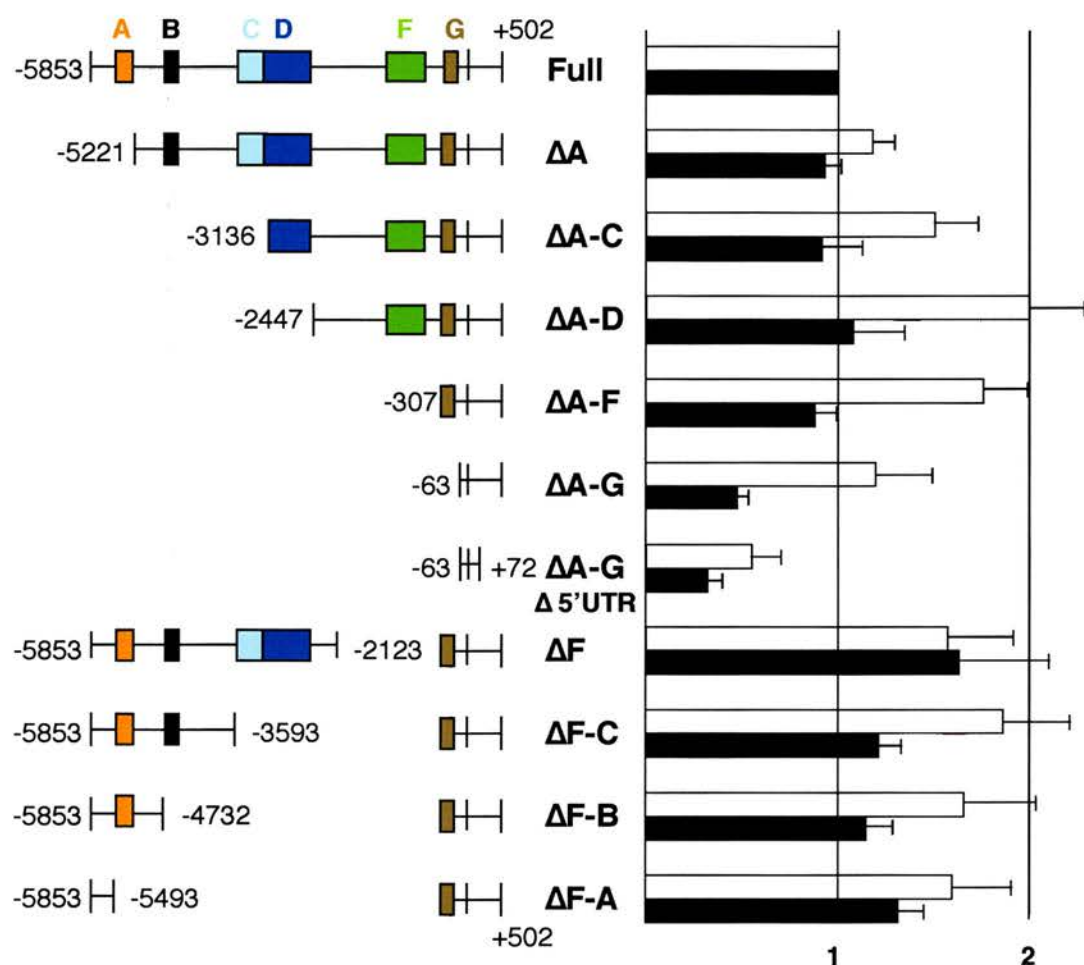


Fig. 6.6 Deletion analysis of the *Fgf10* promoter to identify regulatory elements.

Promoter constructs were cloned into pA3LUC, a luciferase expressing vector, and used in transfection studies. Constructs contain the following regions of *Fgf10* promoter sequence: Full, 5.8kb; ΔA, 5.2kb; ΔA-C, 3.1kb; ΔA-D, 2.4kb; ΔA-F, 307bps; and ΔA-G, 63bps. ΔA-G Δ5'UTR, 63bps of DNA 5' and 72bps of DNA 3' to the transcription start site. ΔF, ΔF-C, ΔF-B and ΔF-A represent internal 5' DNA deletions from -63 of 2.1kb, 3.5kb, 4.7kb and 5.4kb, respectively. Promoter constructs were co-transfected with a βgal expressing vector into NIH-3T3 (clear bars) and VMP cells (solid bars) and cultured in CPSR-containing media for 36hrs. Results represent luciferase values normalised for transfection efficiency (by βgal assay) and standardised to the Full length promoter luciferase activity. NIH-3T3 cells, 5' to 3' and internal deletions showed no significant change in activity between constructs. A two-fold down-regulation was observed in ΔA-G Δ5'UTR. VMP cells, 5' to 3' deletions up to ΔA-F and internal deletions showed no significant change in activity between constructs. ΔA-G and ΔA-G Δ5'UTR deletions resulted in a two-fold down-regulation of activity. Error bars represent SEM.

deletions up to $\Delta A-D$, and gradually decreased in activity with deletions up to $\Delta A-G$ $\Delta 5'$ UTR. Internal deletions showed no significant change in activity between constructs in NIH3T3 cells. In primary VMPC, 5' to 3' deletions up to $\Delta A-F$, and internal deletions showed no significant change in activity between constructs. $\Delta A-G$ and $\Delta A-G$ $\Delta 5'$ UTR deletions resulted in a two-fold down-regulation of activity. As the effects observed in primary VMPC were probably more representative of the effect in the prostate than in NIH3T3 cells, region G was further investigated.

6.2.5 Investigation of Region G

Region G was examined for transcription factor binding sites using GeneJockeyII and for promoter regions using NIX analysis. NIX analysis is intended as a tool to aid the identification of interesting regions in Genomic or transcribed nucleic acid sequences and is found on the website <http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/>. GeneJockeyII identified a consensus Sp1 site in region G. Also the unusually high G+C content of the 5' flanking region to the transcription start site suggested the presence of a CpG island. To test if this region would qualify as a CpG island the sequence was run through NIX analysis. A CpG island was predicted starting at -391 and finishing at -117, containing 62 % G + C content (Fig. 6.3). This region included the 5' end of region G and the Sp1 site. CpG islands allow for easy access of transcription factors (Bird, 1986) increasing the possibility that the Sp1 site was relevant to the regulation of *Fgf10* gene expression. Additionally other reports have shown that consensus Sp1 binding sites can mediate TGFbeta1 action (Botella et al., 2001; Brodin et al., 2000; Datta et al., 2000; Li et al., 1995). *Fgf10* promoter constructs were made to investigate both the effect of the Sp1 site on the activity of *Fgf10* promoter constructs and to determine whether the effect of TGFbeta1 on *Fgf10* transcript levels is mediated via the Sp1 site (Fig. 6.7). Constructs were made by PCR and cloning into pA3Lucm (described in section 2.2.6.2. and Tables 2.3 and 2.4). Construct +Sp1 contained the Sp1 site and the sequence 3' to the Sp1 site. Construct Δ Sp1 contained only sequence 3' to the Sp1 site. Constructs were also made to determine whether deletion of the Sp1 site

from within the *Fgf10* sequence would result in any change in activity and if TGFbeta1 would mediate its affect on *Fgf10* transcription through this site. As the activity of ΔA-D was similar to the activity of the full-length construct, it was decided to delete the Sp1 site from ΔA-D. Construct ΔA-D ΔSp1 was made by deleting the Sp1 site from ΔA-D. These promoter constructs plus the full length, ΔA-D, and ΔA-G (core promoter) were co-transfected into primary VMPC with a β-gal expressing vector. Cells were cultured for 36hrs and treated with and without TGFbeta1 for 8hrs (Fig. 6.8). TGFbeta1 treatment resulted in approximately two-fold down-regulation of activity in the Full, ΔA-D and +Sp1 promoter constructs. No down-regulation was observed in the ΔA-D ΔSp1, ΔSp1 and core promoter constructs, with TGFbeta1 treatment. Deletion of the Sp1 site from ΔA-D resulted in a down-regulation of activity, similar to levels in the core promoter. In the absence of TGFbeta1 slightly less activity was observed from ΔSp1 than +Sp1.

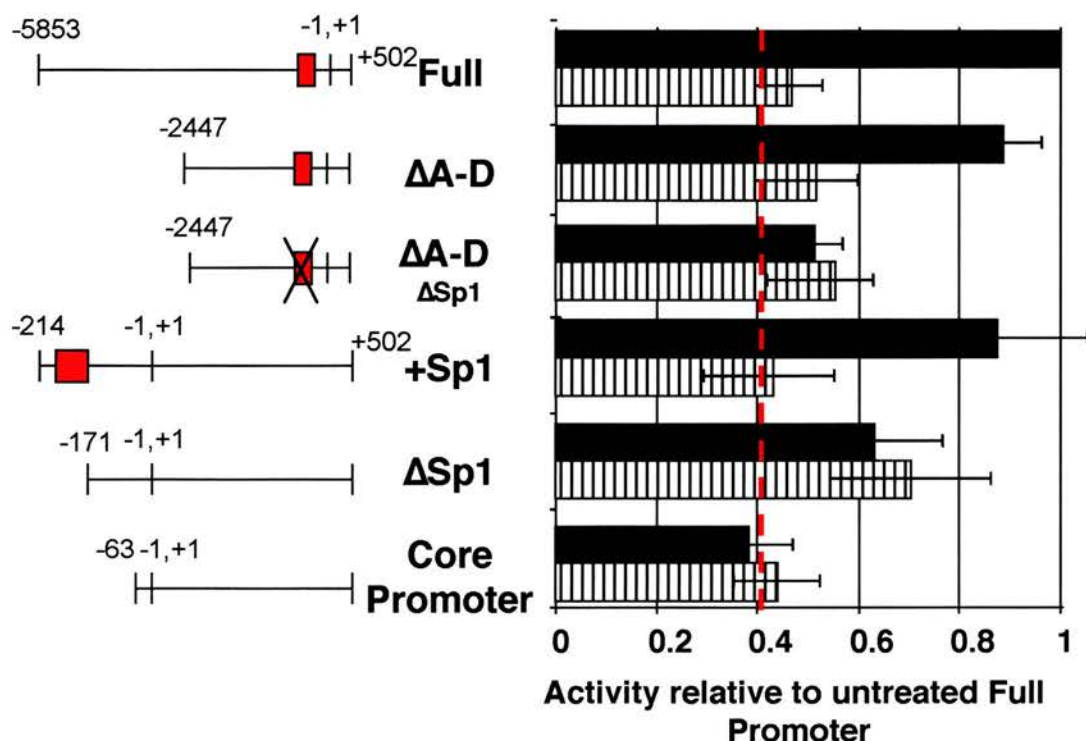


Fig. 6.8 Transfection of pA3LUC promoter constructs to define regions that mediate regulation by TGFbeta1.

Promoter constructs were co-transfected with a βgal expressing vector into primary VMPC. Cells were cultured for 36hrs in CPSR-containing media, and treated with (vertical lined bars) and without (solid bars) TGFbeta1 (5ng/ml) for 8hrs. Results represent luciferase values normalised for transfection efficiency (by βgal assay) and standardised to the full length promoter luciferase value. The average core promoter activity is represented as a red dotted line running down the graph. TGFbeta1 treatment resulted approximately two-fold down-regulation of activity in the Full, ΔA-D and +Sp1 promoter constructs. No down-regulation was observed in the ΔA-D ΔSp1, ΔSp1 and core promoter constructs when treated with TGFbeta1. Deletion of the Sp1 site from ΔA-D resulted in a down-regulation of activity, similar to levels in the core promoter. Deletion of the Sp1 site from +Sp1 resulted in a small decrease in activity, intermediate between full length and core promoter. Error bars represent SEM.

6.3 Summary of Results

Promoter analysis was performed to understand the mechanisms responsible for regulating *Fgf10* gene expression. A comparison of human and mouse genomic sequences 5' to the *Fgf10* exon1 and sheep cDNA sequence revealed a sharp dip in homology around 1300 nucleotides 5' of the translation start site. The *Fgf10* transcription start site was mapped by RNase protection assay. The presence of several protected bands suggested there was more than one transcription start site for *Fgf10*. Amongst these bands, a single band was consistently stronger than others with every probe used, this probably represented the major transcription start site for *Fgf10*. Analysis of the *Fgf10* promoter revealed that it is a TATA-less promoter. Characteristic features of TATA-less are the presence of an initiator element (Inr), upstream Sp1 sites and downstream promoter elements (DPE). Consensus regions containing these sites were identified in the *Fgf10* promoter and are shown on Fig. 6.3.

A comparative analysis of mouse and human *Fgf10* genomic sequences revealed that many regions were highly conserved between human and mouse *Fgf10* promoter sequences. This suggests that these regions may contain sites that control *Fgf10* gene expression. To examine if these regions regulated *Fgf10* gene expression two approaches were adopted; enhancer assays and promoter deletions. Region A (-5492, -5222) was shown to enhance the activity of an SV40 promoter vector and region F (-1265, -667) was shown to repress the activity. Region F also inhibited the increase in activity caused by region A, suggesting that region F could repress *Fgf10* transcription caused by an enhancer. To determine if the regions identified by the promoter assay regulated the *Fgf10* core promoter, deletion constructs of the *Fgf10* promoter were constructed in a luciferase-expressing vector. Deletion of region A or region F did not significantly change the activity of the *Fgf10* core promoter, suggesting that both regions did not play a significant part in the regulation of *Fgf10* gene expression. However, deletion of region G resulted in a two-fold down-regulation of *Fgf10* activity in primary

VMPC, although region G had no enhancer activity in SV40 assay. The difference in results may be an artefact of the cellular *in vitro* system. However, as the enhancer assay uses a core promoter that is different to that of *Fgf10*, probably the most accurate interpretation of these results would be gained from using the *Fgf10* core promoter and further investigation of region G. Region G incorporated some of the putative CpG island, highly suggestive that this region does contain a transcription factor-binding site that regulates *Fgf10* gene expression. A putative Sp1 binding site was identified using a transcription factor binding site search by the computer program GeneJockeyII. Sp1 binding sites have been shown to mediate the effect of TGFbeta1 in some circumstances (Botella et al., 2001; Li et al., 1995). Hence the repression of *Fgf10* gene expression by TGFbeta1, described in earlier chapters, might be mediated through the Sp1 site. To test this theory the Sp1 site was deleted from promoter constructs and the effect of TGFbeta1 on promoter construct activity was tested. Deletion of the Sp1 site repressed the activity of the *Fgf10* promoter to levels similar to the activity observed in the *Fgf10* core promoter. Also TGFbeta1 repressed the activity of the promoter in constructs containing Sp1 sites but had no effect on promoter constructs that had the Sp1 site removed. The activity of *Fgf10* promoters treated with TGFbeta1 was similar to the activity in the *Fgf10* core promoter. These results suggested that the Sp1 site promotes *Fgf10* gene expression and TGFbeta1 represses *Fgf10* gene expression through the Sp1 site.

7. Regulation of VP Growth by TGFbeta1

7.1 Introduction

TGFbeta isoforms have been shown to play a role in prostatic regression after androgen deprivation (Kyprianou and Isaacs, 1988; Kyprianou and Isaacs, 1989; Martikainen et al., 1990), and TGFbeta1 has been postulated as being an important factor in stromal-epithelial interactions during prostate and seminal vesicle development (Itoh et al., 1998a; Tanji et al., 1994). All three TGFbeta peptides have been localised to prostatic epithelium and stroma by immunostaining (Perry et al., 1997; Timme et al., 1994) and their transcripts have been detected in cultured prostate stromal and epithelial cells (Itoh et al., 1998a; Story et al., 1996). TGFbeta1 inhibits proliferation of many normal and transformed cell types (Massague et al., 1992b), including prostatic epithelial cells (McKeehan and Adams, 1988), but it has been shown to increase proliferation in some human cancer cell types (Hsu et al., 1994; Kansra et al., 2000; Lamm et al., 1998; Schroy et al., 1990).

Organ culture of P0 rat VPs was used to address the possible physiological function of TGFbeta1 on prostate development. Experiments were designed to determine if TGFbeta1 affected the size, prostatic bud number, and proliferation rate of epithelial and stromal cells in the VP. Also the effect of culturing organ rudiments with testosterone and TGFbeta1 on *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* gene expression was investigated.

7.2 Results

7.2.1 The Effect of TGFbeta1 on VP Size

P0 VPs were cultured in the presence and absence of TGFbeta1 and testosterone (Fig. 7.1) to address the potential physiological function of TGFbeta1. Whole mount live images of VPs were captured at the end of culture (Fig. 7.1A). The images were imported into NIH Image 1.62f, and the two-dimensional area of VPs was measured and represented as pixels x 1000. The sizes of the organs were as follows; -T, 95 pixels; -T+TGFbeta1, 75 pixels; +T, 151 pixels; +T+TGFbeta1, 103 pixels. TGFbeta1 inhibited 86 % of testosterone-induced growth. TGFbeta1 reduced the size of organs grown -T by 21 % and reduced the size of organs grown +T by 32 %. The two-dimensional area of VPs cultured with TGFbeta1 was significantly lower both in the presence and absence of testosterone after six days (-T+TGFbeta1 21% reduction compared to -T, Student's t test $P=4.79e^{-4}$; +T+TGFbeta1 32% reduction compared to +T, Student's t test $P=4.03e^{-9}$)(Fig. 7.1B). This suggests that TGFbeta1 inhibits prostatic growth and development. This also suggests that testosterone does not inhibit the effects of TGFbeta1 on VP growth although ligand bound AR has been previously shown to inhibit TGFbeta mediated transcriptional responses (Chipuk et al., 2002).

7.2.2 The Effect of TGFbeta1 on Epithelial Bud Number

Another way in which the physiological function of TGFbeta1 was addressed was by counting the number of epithelial buds (Fig. 7.2). Previous research on the effect of TGFbeta1 on branching morphogenesis of VPs counted the number of bud per mm^3 over the whole organ (Itoh et al., 1998a), but this was almost impossible in our system as individual buds within the VP images were hard to distinguish. It was decided that an easier way to tell if TGFbeta1 affected bud number would be to count the number of buds around the periphery of the organs (Fig. 7.2A). The number of buds was expressed

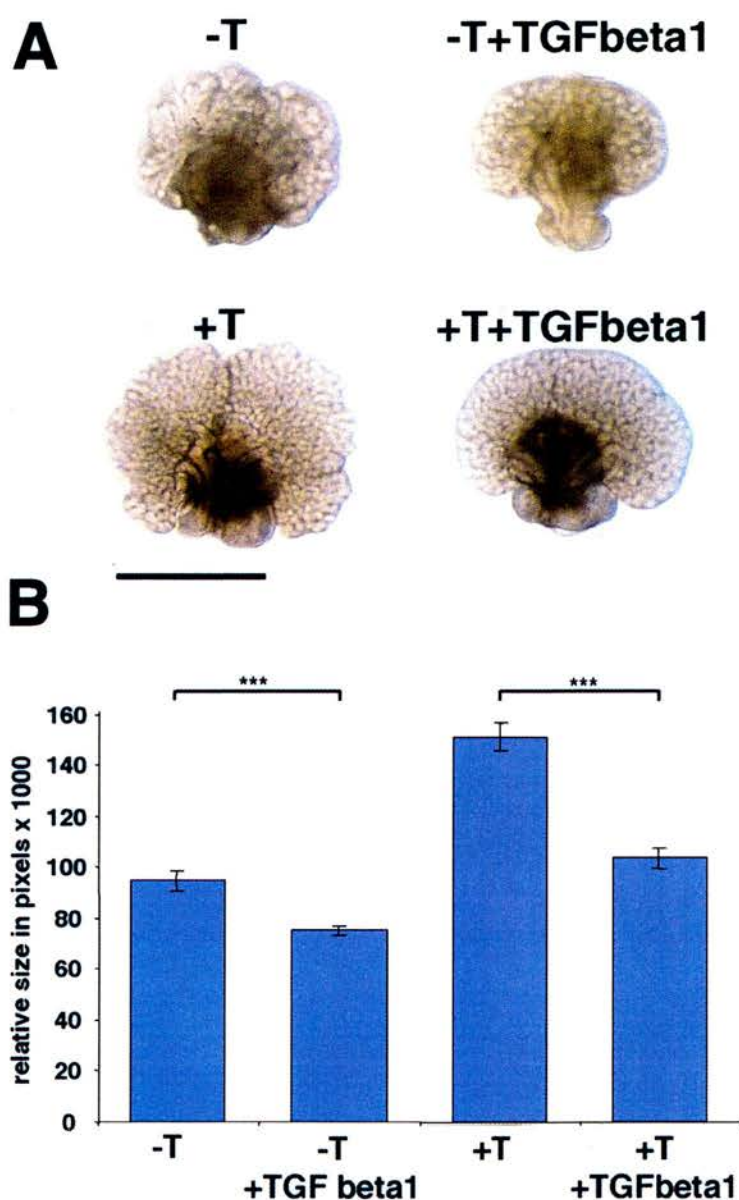


Fig. 7.1 The effect of TGFbeta1 on VP size.

P0 rat VPs were cultured for six days in the presence or absence of testosterone (10^{-8} M) and/or TGFbeta1 (10ng/ml) (scale bar = 1mm). Organs were imaged under lightfield illumination at the end of culture prior to fixation or freezing for RNA. The data represents 30 organs from each treatment group from 4 independent experiments. Panel A. Representative pictures of cultured VPs. Panel B. The two dimensional area of VPs was measured using NIH-image analysis; area represented as pixels was calculated and plotted on a graph. Addition of TGFbeta1 to VPs cultured in the presence and absence of testosterone resulted in a significant reduction in area (-T+TGF 20% reduction compared to -T, Student's t test $P=4.79e^{-4}$; +T+TGF 30% reduction compared to +T, Student's t test $P=4.03e^{-9}$).

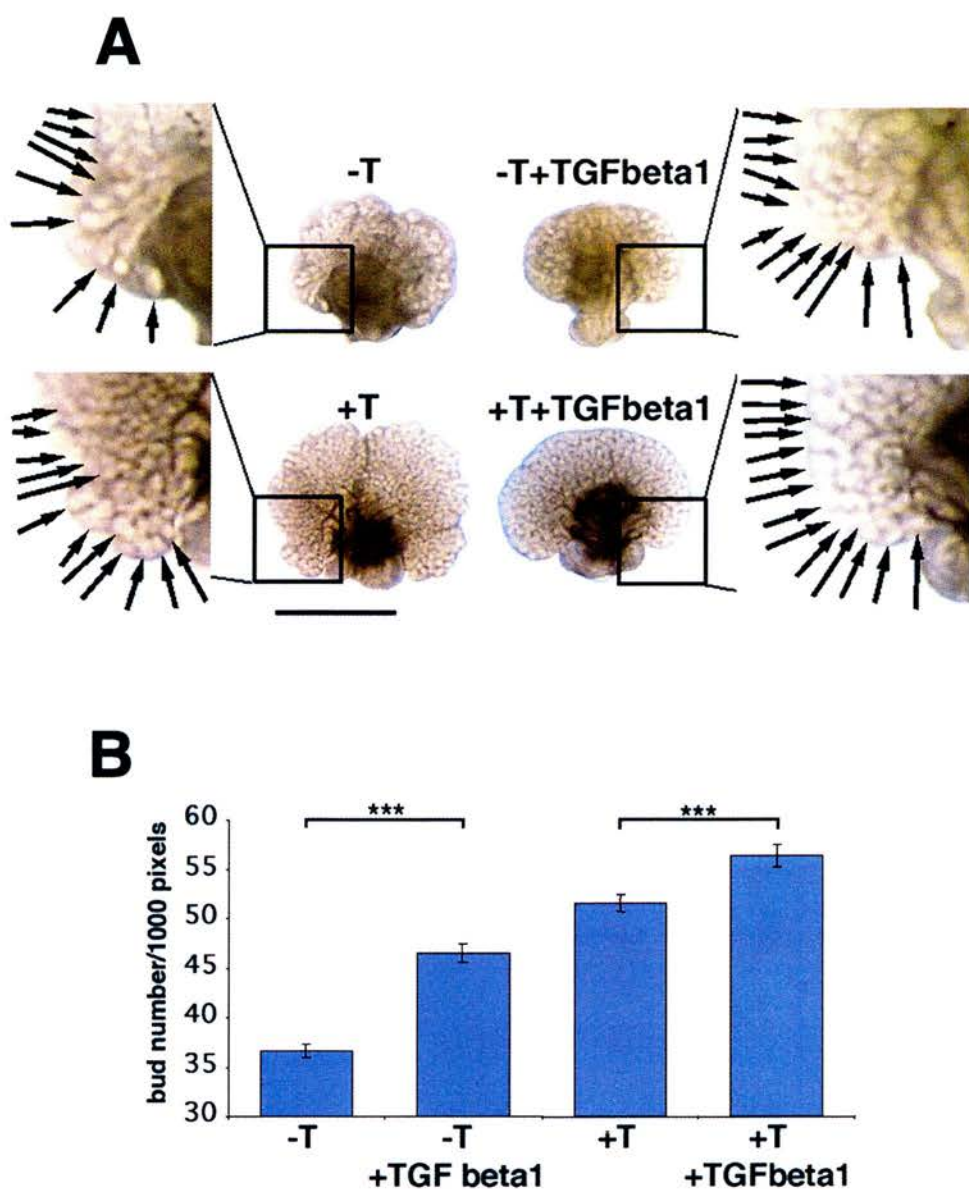


Fig. 7.2 The effect of TGFbeta1 on epithelial bud number.

VPs from P0 rats were grown for six days in the presence or absence of testosterone (10^{-8} M) and/or TGFbeta1 (10ng/ml). 30 organs from 4 independent experiments were measured using NIH-image. Panel A. Representative pictures of cultured VPs (scale bar = 1mm). The VPs were magnified to demonstrate bud number. Panel B. The number of epithelial bud tips around the periphery of organs (shown with arrows, panel A) from each treatment were counted and shown as bud number per 1000 pixels of perimeter. The addition of TGFbeta1 to organs cultured in the absence or presence of testosterone caused significant increases in the number of epithelial bud tips (-T compared to -T+TGF, Student's t test $P=7.89e^{-6}$; +T compared to +T+TGF, Student's t test $P=8.30e^{-4}$).

as a ratio to organ perimeter (bud number/1000 pixels of perimeter (using NIH Image)) so that organs of different sizes could be compared (Fig. 7.2B). The number of buds per 1000 pixels of perimeter were; -T, 36.6; -T+TGFbeta1, 46.6, +T, 51.7; +T+TGFbeta1, 56. TGFbeta1 significantly increased the number of buds/1000 pixels of perimeter in VPs cultured with and without testosterone (-T compared to -T+TGFbeta1, Student's t test $P=7.89e^{-6}$; +T compared to +T+TGFbeta1, Student's t test $P=8.30e^{-4}$). Again this suggests that testosterone does not inhibit the effects of TGFbeta1 on VP growth and budding.

So, although the two dimensional area of VPs growth with TGFbeta1 is decreased, the number of buds in the periphery of the organs is increased, suggesting that TGFbeta1 may have differential effects on the proliferation rate of cells within the organ. To determine if this was occurring the effect of TGFbeta1 on the proliferation rate of cells in VPs was investigated.

7.2.3 The Effect of TGFbeta1 on VP Cell Proliferation

TGFbeta has been demonstrated to have differing effects on the proliferation rate of cells from different lineages (Massague et al., 1992b). The aim of this part of the thesis was to determine the effect of TGFbeta1 on VP cell proliferation, and to determine whether any changes in cell proliferation correlated with the changes in organ size when treated with TGFbeta1. The effect of TGFbeta1 on proliferation of epithelial and stromal cells was examined in P0 VP organ cultures. VPs were cultured for either three days or six days to examine the effect of TGFbeta1 at different stages of the culture system. BrdU was added on the final day of culture, and was detected by immunohistochemistry and visualised by both fluorescent illumination and con-focal microscopy. Proliferation rate was measured by counting the number of cells that had incorporated BrdU and represented as a percentage to total cell number (Table 7.1).

Table 7.1 showing the percentage BrdU incorporation in proximal and distal cells to urethra after culture with and without T or TGFbeta1 for 3 or 6 days

Treatment	3 day				6 day			
	Epithelia (% BrdU)		Stroma (% BrdU)		Epithelia (% BrdU)		Stroma (% BrdU)	
	Proximal	Distal	Proximal	Distal	Proximal	Distal	Proximal	Distal
-T	20.8+/-1.4	33.8+/-2.5	16.3+/-0.8	22.8+/-1.4	10.4+/-0.9	13+/-1.1	7.8+/-0.6	9.6+/-1.4
-T +TGFbeta1	13.2*+/-0.9	40.9+/-2.0	10.1*+/-0.9	21+/-1.3	7.3*+/-0.6	24.6*+/-0.7	3.9*+/-0.5	13.4*+/-0.7
+T	25.6+/-0.9	45.1+/-2.6	15.4+/-1.0	22.3+/-1.0	7.3+/-0.8	26.4+/-1.4	5.9+/-1.0	12.5+/-1.1
+T +TGFbeta1	17.8*+/-1.0	47.1+/-1.3	9.3*+/-1.1	24.7+/-0.7	2.4*+/-0.5	26.3+/-1.1	1.8*+/-0.6	10.8+/-1.0

*represents significant difference compared to relevant treatment without TGFbeta1

7.2.3.1 Three-Day Culture

Lower incorporation of BrdU was observed in the area proximal to urethra in VPs cultured with TGFbeta1 and testosterone for three days (Fig. 7.3, Table 7.1). In the *distal* region to urethra (Fig. 7.4A), TGFbeta1 did not significantly change epithelial or stromal proliferation in the absence or presence of testosterone after three days (Fig. 7.4B) although a small increase in the proliferation of epithelial cells was apparent. However, in the *proximal* region TGFbeta1 significantly reduced epithelial and stromal proliferation in the VP both in the presence and absence of testosterone (Fig. 7.4C,D) (epithelium: -T compared to -T+TGFbeta1, Student’s t test $P=5.70e^{-6}$; +T compared to +T+TGFbeta1, Student’s t test $P=1.45e^{-7}$) (stroma: -T compared to -T+TGFbeta1, Student’s t test $P=1.19e^{-7}$; +T compared to +T+TGFbeta1, Student’s t test $P=2.11e^{-5}$). This suggested that during the first three days of culture TGFbeta1 decreased the proliferation rate of proximal stromal and epithelial cells, but did not significantly affect the proliferation rate of distal epithelia and stroma. Hence, the VPs two-dimensional area would be smaller after six-days as observed in a previous result (Fig. 7.1). Next we assessed whether the proliferation rate of cells in the proximal and distal regions (to urethra) changed in VPs cultured for six days.

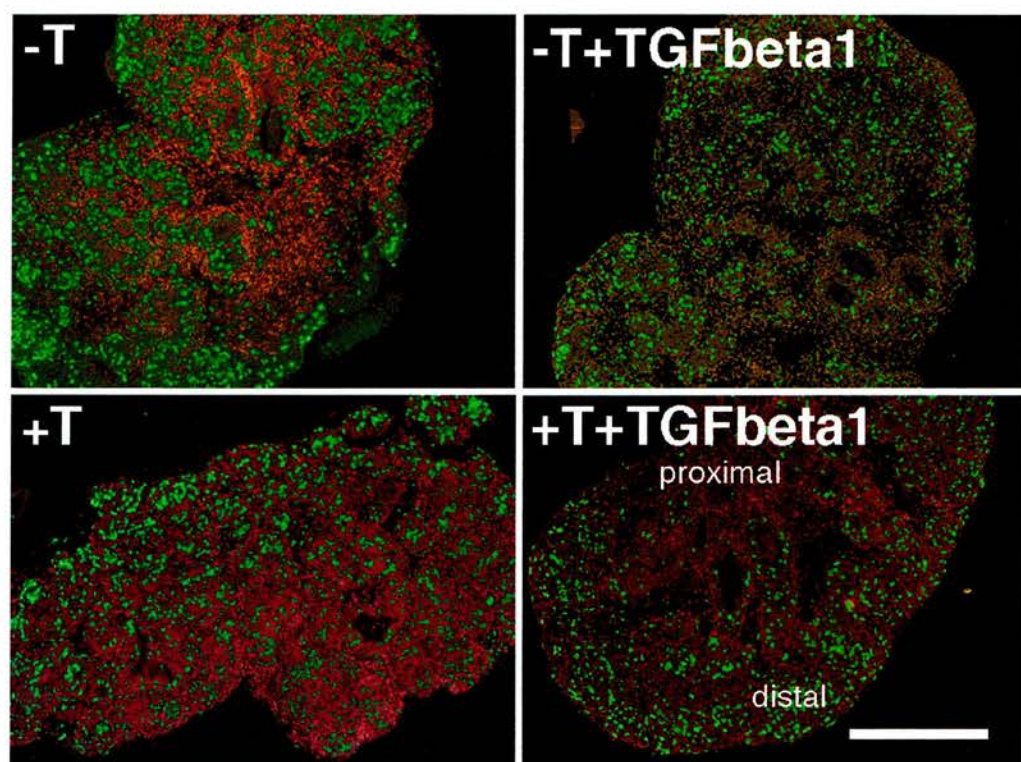


Fig. 7.3 The effect of TGFbeta1 on cell proliferation in VPs on day 3 of culture.

VPs were cultured for three days in the presence or absence of testosterone (10^{-8} M) and/or TGFbeta1 (10ng/ml). BrdU was added on day three of culture. Incorporation of BrdU was detected by immunohistochemistry (green) and the nuclei were counterstained with propidium iodide (red). Immunohistochemistry was detected under fluorescent illumination at X10 magnification (scale bar = $500\mu\text{m}$). This was performed on 8 organs from 3 independent experiments. VPs cultured with TGFbeta1 in the presence of testosterone resulted in lower BrdU incorporation in the area proximal to urethra. No change in BrdU incorporation was obvious at this magnification in VPs cultured with TGFbeta1 in the absence of testosterone.

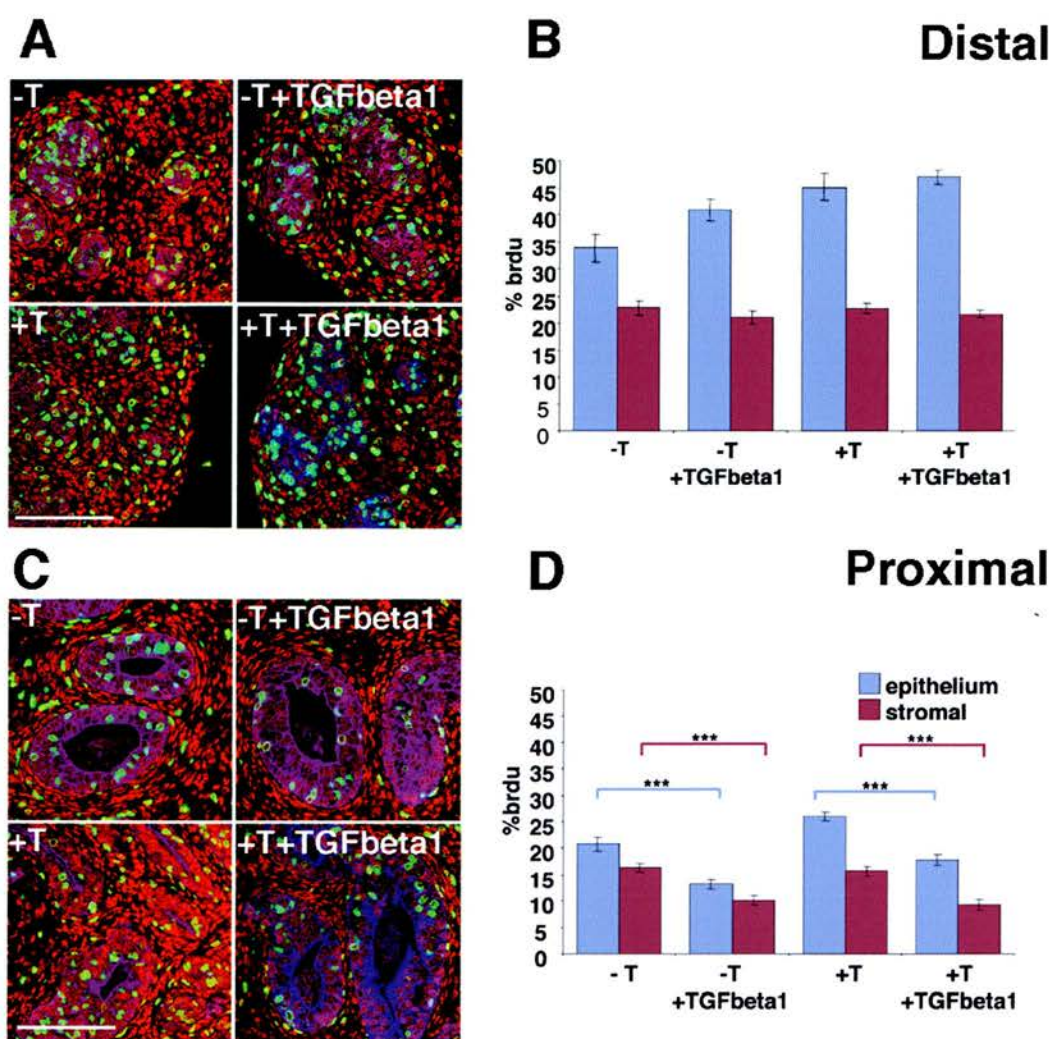


Fig. 7.4 The effect of TGFbeta1 on proliferation of epithelial and stromal cells in distal and proximal regions of VPs cultured for 3days

VPs were cultured in the presence and absence of testosterone (10^{-8} M) and/or TGFbeta1 (10 ng/ml). Proliferation rate was measured by calculating the percentage of cells that incorporate BrdU, added on day three of culture. Incorporation of BrdU in distal (A) and proximal (C) regions to urethra were detected by immunohistochemistry (green, yellow when co-localised to nuclei) and epithelial cells were distinguished by immunohistochemistry for pan-Cytokeratin (blue). Nuclei were counterstained with propidium iodide (red) (scale bar = 100 μ m). Panel B. Data from the **distal** region to urethra representing 15 measurements on 8 organs from each treatment group from three independent experiments. TGFbeta1 did not significantly change epithelial or stromal proliferation in the absence or presence of testosterone in the distal region (-T compared to -T+TGF and +T compared to +T+TGF, Student's t-test $P > 0.005$). Panel D. Data from the **proximal** region to urethra representing 18 measurements on 9 organs from each treatment group from three independent experiments. TGFbeta1 significantly reduced epithelial (-T compared to -T+TGF, Student's t-test $P = 3.15 \times 10^{-3}$; +T compared to +T+TGF, Student's t-test $P = 2.83 \times 10^{-6}$) and stromal (-T compared to -T+TGF, Student's t-test $P = 1.38 \times 10^{-5}$; +T compared to +T+TGF, Student's t-test $P = 4.63 \times 10^{-4}$) proliferation both in the presence and absence of testosterone.

7.2.3.2 Six-Day Culture

VPs cultured with TGFbeta1 in the presence and absence of testosterone resulted in a lower BrdU incorporation in epithelial and stromal cells in the area proximal to urethra (Fig. 7.5 and Table 7.1). In the distal region TGFbeta1 significantly increased epithelial and stromal proliferation in the *absence* of testosterone (Fig. 7.6A,B) (epithelium: -T compared to -T+TGFbeta1, Student's t test $P=1.11e^{-7}$) (stroma: -T compared to -T+TGFbeta1, Student's t test $P=2.49e^{-5}$). TGFbeta1 had no significant effect on epithelial or stromal proliferation in the *presence* of testosterone (Fig. 7.6A,B). This may be due to testosterone already stimulating cell growth maximally; therefore TGFbeta1 may not be able to induce any further increase in proliferation. In the proximal region TGFbeta1 significantly reduced epithelial and stromal proliferation both in the presence and absence of testosterone (Fig. 7.6C,D) (epithelium: -T compared to -T+TGFbeta1, Student's t test $P=3.15e^{-3}$; +T compared to +T+TGFbeta1, Student's t test $P=2.83e^{-6}$) (stroma: -T compared to -T+TGFbeta1, Student's t test $P=1.38e^{-5}$; +T compared to +T+TGFbeta1, Student's t test $P=4.63e^{-4}$). Testosterone also reduced the level of BrdU incorporation in the proximal region compared to untreated organs, and the addition of testosterone and TGFbeta1 caused a greater repression of proliferation.

Hence the effects of TGFbeta1 on proliferation were not inhibited by testosterone suggesting the changes in VP growth may be due to changes in the proliferation rate of cells, and that TGFbeta1 had differential effects on cell proliferation in different regions of the VP.

7.2.4 Analysis of *Tgfbeta* Transcript Levels

Next it was decided to investigate *Tgfbeta* gene regulation in neonatal prostatic rudiments, as numerous studies have examined the regulation of *Tgfbeta* transcript levels in adult prostates and prostate cells. In the adult, testosterone negatively regulates the

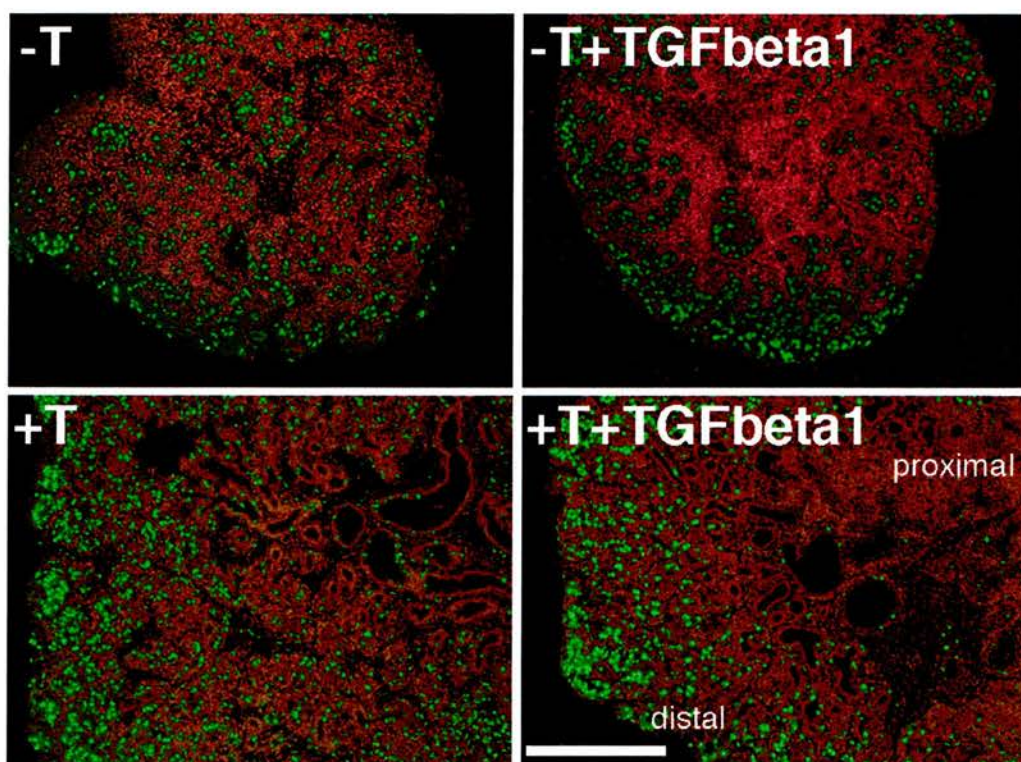


Fig. 7.5 The effect of TGFbeta1 on cell proliferation in VPs on day 6 of culture. VPs were cultured for six days in the presence or absence of testosterone (10^{-8} M) and/or TGFbeta1 (10ng/ml). BrdU was added on day six of culture. Incorporation of BrdU was detected by immunohistochemistry (green) and the nuclei were counterstained with propidium iodide (red). Immunohistochemistry was detected under fluorescent microscopy (scale bar = $500\mu\text{m}$). This was performed on 9 organs from 3 independent experiments. VPs cultured with TGFbeta1 in the presence or absence of testosterone resulted in a lower BrdU incorporation in the area proximal to the urethra of VPs.

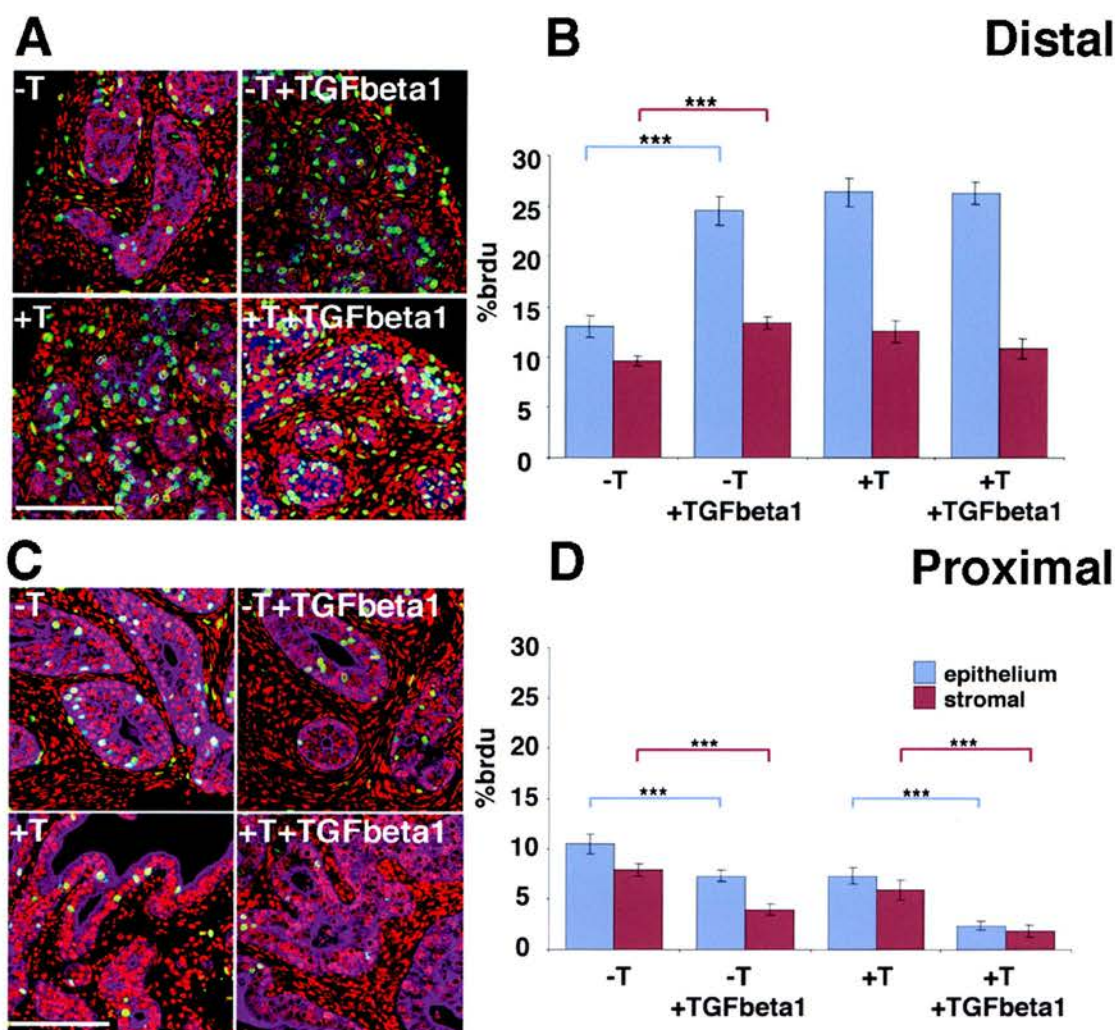


Fig. 7.6 The effect of TGFbeta1 on proliferation of epithelial and stromal cells in VPs cultured for 6 days.

VPs were cultured in the presence or absence of testosterone (10^{-8} M) and/or TGFbeta1 (10ng/ml). Proliferation rate was measured by calculating the percentage of cells that incorporated BrdU added on day 6 of culture. Incorporation of BrdU in the distal (A) and proximal (C) regions were detected by immunohistochemistry (green, yellow when co-localised with nuclei) and epithelial cells were distinguished by immunohistochemistry for pan-Cytokeratin (blue). Nuclei were counterstained with propidium iodide (red) (scale bar = $100\mu\text{m}$). The data represents 18 measurements on 9 organs from each treatment group from 3 independent experiments. Panel B **Distal** region to urethra. TGFbeta1 significantly increased epithelial (-T compared to -T+TGF, Student's t-test $P=1.11\text{e}^{-7}$) and stromal (-T compared to -T+TGF, Student's t-test $P=2.49\text{e}^{-5}$) proliferation in the absence of testosterone. TGFbeta1 had no significant effect on epithelial or stromal proliferation in the presence of testosterone. Panel D. **Proximal** region to urethra TGFbeta1 significantly reduced epithelial (-T compared to -T+TGF, Student's t-test $P=3.15\text{e}^{-3}$; +T compared to +T+TGF, Student's t-test $P=2.83\text{e}^{-6}$) and stromal (-T compared to -T+TGF, Student's t-test $P=1.38\text{e}^{-5}$; +T compared to +T+TGF, Student's t-test $P=4.63\text{e}^{-4}$) proliferation both in the presence and absence of testosterone.

expression of TGFbeta ligands (Bacher et al., 1993; Itoh et al., 1998a; Kyprianou and Isaacs, 1989). TGFbeta1 has been shown to positively regulate its own expression in normal and transformed cells (Van Obberghen-Schilling et al., 1988) however, auto-regulation was not observed in primary prostatic stromal and epithelial cells (Itoh et al., 1998a). The effect of testosterone and TGFbeta1 was examined in VPs (Fig. 7.7) and VMP+UR (Fig 7.8) in organ culture. The long and short-term effects of TGFbeta1 on *Tgfbeta* transcript levels were examined. P0 VPs were cultured either for six days in the presence or absence of testosterone and/or TGFbeta1 (Fig. 7.7A), or for three days in the presence or absence of testosterone, then for seven hours with or without TGFbeta1 (Fig. 7.7B). VMP+UR were cultured either for three days in the absence or presence of testosterone and/or TGFbeta1 (Fig. 7.8A) or for three days in serum-free conditions then treated for seven hours in the presence and absence of testosterone and/or TGFbeta1 (Fig. 7.8B). VMP+UR were cultured only for three days because a six-day culture causes them to appear necrotic, so it was hard to compare the regulation of *Tgfbeta* transcript levels in male and female organ rudiments.

7.2.4.1 Analysis of *Tgfbeta* Transcript Levels in VPs

No change in *Tgfbeta* transcript levels was observed in VPs cultured for six days in the presence or absence of testosterone and/or TGFbeta1 (Fig. 7.7A and table 7.2). *Tgfbeta2* transcript levels were lower in VPs cultured with testosterone for three days both in the presence and absence of TGFbeta1 for seven hours (Fig. 7.7B). *Tgfbeta1* and *Tgfbeta3* transcript levels were higher in VPs cultured with TGFbeta1 for seven hours, in the absence of testosterone, but not in the presence of testosterone. This suggests that testosterone inhibited the TGFbeta1 induced increase of *Tgfbeta1* and *Tgfbeta3* transcript levels.

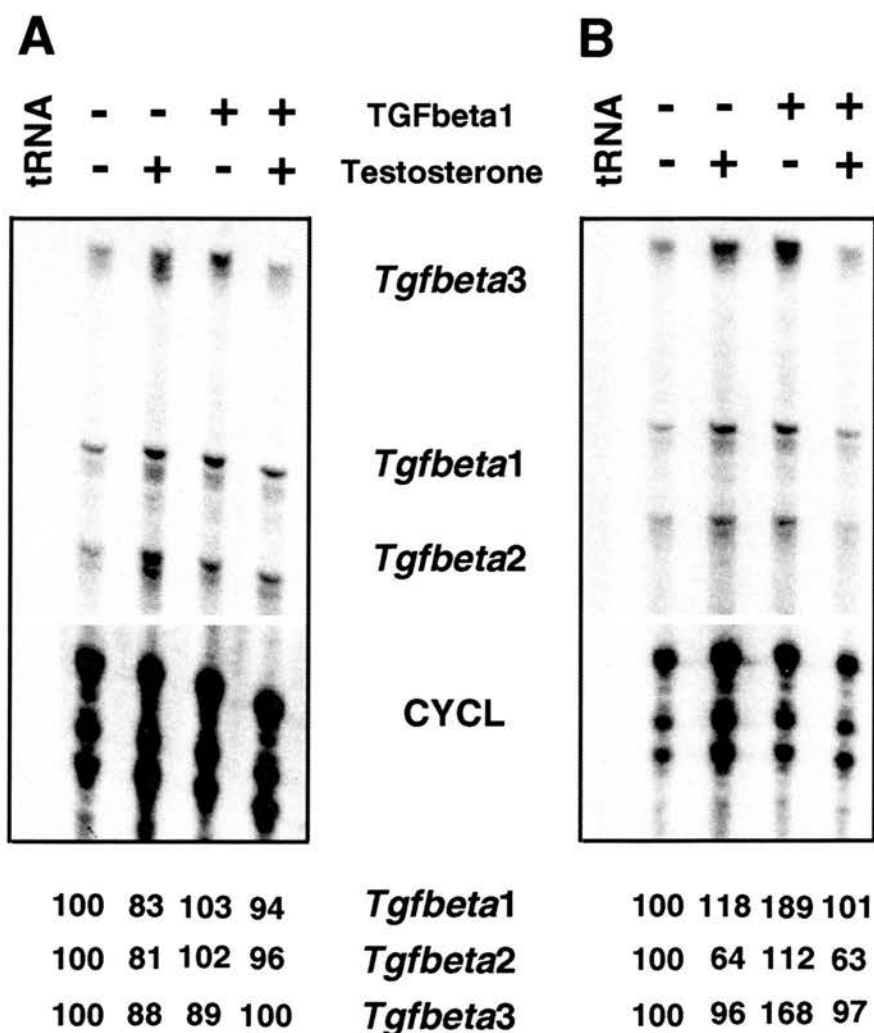


Fig. 7.7 Analysis of *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* transcript levels in VPs. P0 VPs were cultured in the presence and absence of TGFbeta1 (10ng/ml) and/or testosterone (10^{-8} M) for six days (panel A). Also, VPs were cultured for three days in the presence and absence of testosterone (10^{-8} M) then treated for seven hours in the presence or absence of testosterone and/or TGFbeta1 (10ng/ml) (Panel B). VP *Tgfbeta* transcripts were assayed by RNase protection, quantified using a phosphoimager and normalised to cyclophilin (CYCL). The numbers below panel A and B represent the average transcript abundance of *Tgfbeta* isoforms relative to levels in VPs cultured in the absence of testosterone and TGFbeta1 (n=2). No changes in *Tgfbeta* transcript levels were observed in VPs cultured for 6 days. A 3 day testosterone treatment caused a small decrease in *Tgfbeta2* transcript levels in the presence and absence of TGFbeta1. A 7hr TGFbeta1 treatment in the absence of testosterone caused a 1.9- and 1.7-fold increase in *Tgfbeta1* and *Tgfbeta3* transcript levels, respectively.

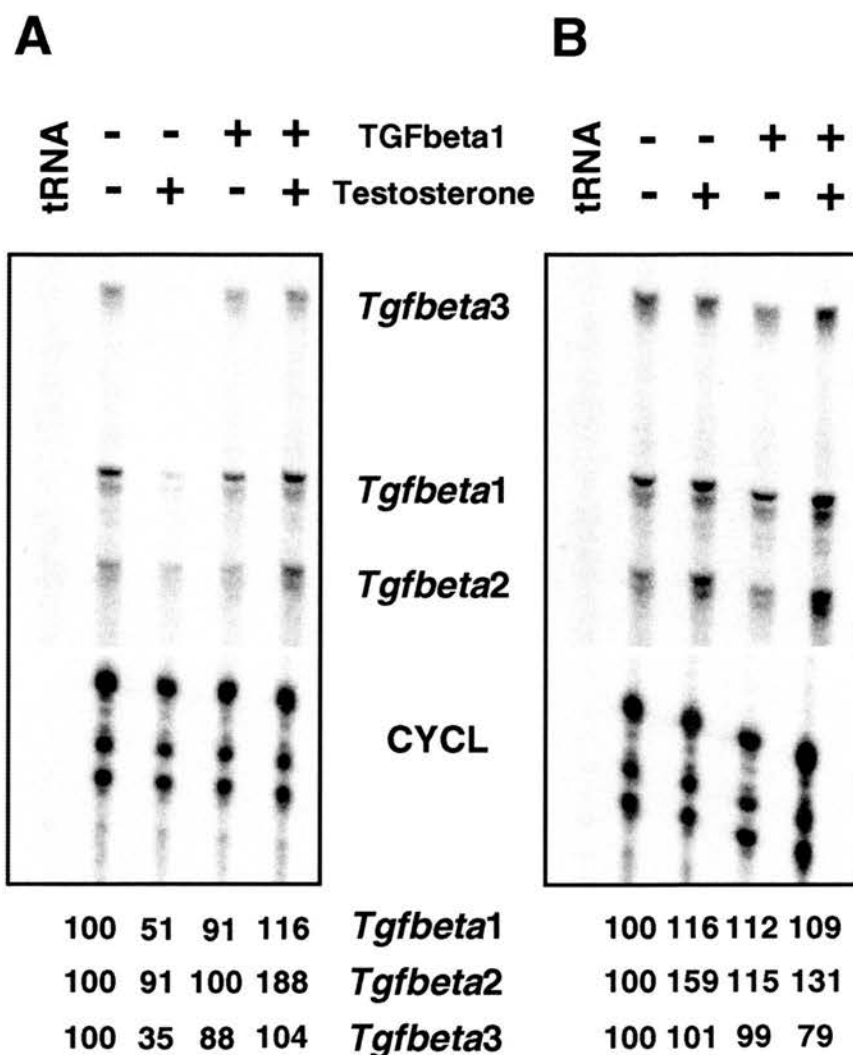


Fig. 7.8 Analysis of *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* transcript levels in P0 female VMP+UR

P0 female VMP+UR were cultured in the presence and absence of TGFbeta1 (10ng/ml) and/or testosterone (10⁻⁸M) for three days (panel A). Also, P0 female VMP+UR were cultured for three days in serum free conditions then cultured for seven hours in the presence or absence of testosterone and/or TGFbeta1 (10ng/ml) (Panel B). VMP+UR *Tgfbeta* transcripts were assayed by RNase protection, quantified using a phosphorimager and normalised to cyclophilin (CYCL). The numbers below panel A and B represent the average transcript abundance of *Tgfbeta* isoforms relative to levels in VMP+UR cultured in the absence of testosterone and TGFbeta1 (n=2). *Tgfbeta1* and *Tgfbeta3* transcript levels decreased by 2- and 3-fold, respectively, when cultured in the presence of testosterone for 3 days. *Tgfbeta2* transcript levels increased by 1.8-fold when cultured in the presence of testosterone and TGFbeta1 for 3 days, and increased by 1.6-fold when cultured in the presence of TGFbeta1 for 7 hrs.

7.2.4.2 Analysis of *Tgfbeta* Transcript Levels in VMP+UR

Tgfbeta1 and *Tgfbeta3* transcript levels were decreased by 2- and 3-fold, respectively, in VMP+UR cultured in the presence of testosterone for 3 days (Fig. 7.8A and table 7.3). However no change in these transcript levels was observed when cultured in the presence of testosterone and TGFbeta1, which suggested that TGFbeta1 inhibited testosterone action. Over three-days' culture, *Tgfbeta2* transcript levels were increased in the presence of testosterone and TGFbeta1 by 1.9-fold. No other changes in *Tgfbeta* transcript levels were observed. A seven-hour testosterone treatment of VMP+UR resulted in a 1.6-fold increase in *Tgfbeta2* transcript levels (Fig. 7.8B). No change in *Tgfbeta1* and *Tgfbeta3* transcript levels was observed over this treatment period.

Table 7.2 Summary of changes in levels of *Tgfbeta* transcripts in VPs

Tgfbeta mRNA	Testosterone			TGFbeta1			Testosterone + TGFbeta1			
	7hrs	3day	6day	7hrs	3day	6day	7hrs	3d ^(T) + 7hrs (TGFbeta1)	3day	6day
1		118	83	189		103		101		94
2		64	81	112		102		63		96
3		96	88	168		89		97		100

Table 7.3 Summary of changes in levels of *Tgfbeta* transcripts in VMPs

Tgfbeta mRNA	Testosterone			TGFbeta1			Testosterone + TGFbeta1			
	7hrs	3day	6day	7hrs	3day	6day	7hrs	3d ^(T) + 7hrs (TGFbeta1)*	3day	6day
1	116	51		112	91		109		116	
2	159	91		115	100		131		188	
3	101	35		99	88		79		104	

* 3 day treatment with testosterone followed by a 7 hr treatment with testosterone and TGFbeta1

7.3 Summary of Results

Experiments were performed to address the function of TGFbeta1 in prostate development, and to determine if testosterone and/or TGFbeta1 regulated the expression of *Tgfbeta* transcripts in prostatic rudiments. To address the effect of TGFbeta1 in prostatic development, VPs were cultured in the presence and absence of TGFbeta1 and/or testosterone for six days. To determine if the changes in VP architecture were due to testosterone and/or TGFbeta regulating *Tgfbeta* transcript levels, VP and VMP+UR were cultured for varying periods of time in the presence and absence of testosterone and/or TGFbeta1. The levels of *Tgfbeta* transcripts were quantified by RNase protection assay.

TGFbeta1 significantly decreased the two-dimensional area of VPs, both in the presence and absence of testosterone. This suggests that TGFbeta1 inhibits prostatic growth and the effect of TGFbeta1 is not inhibited by testosterone. However, surprisingly TGFbeta1 increased the bud number, and increased the proliferation rate of distal (to urethra) epithelia and stroma, in the absence of testosterone, while it inhibited epithelial and stromal proliferation in proximal regions. As epithelial cells are more differentiated in the proximal regions, compared to the distal regions, it could be suggested that TGFbeta1 inhibits proliferation of more differentiated cell types while promoting the proliferation of less differentiated cell types. Also testosterone promotes prostatic cell differentiation in the proximal epithelial cells and the addition of testosterone and TGFbeta1 caused a greater inhibition of proliferation in proximal epithelial cells (67 %) than of those only cultured with TGFbeta1 (30 %). Again this suggests that TGFbeta1 inhibits proliferation of differentiated cells types.

As numerous studies have demonstrated that testosterone and TGFbeta1 can regulate *Tgfbeta* transcript expression the regulation of *Tgfbeta* transcripts by testosterone and TGFbeta1 was investigated. It was demonstrated that the regulation of *Tgfbeta*

transcript levels observed in these studies were similar in the VP or the VMP. These results will be discussed now in reference to previously published data.

An effect of TGFbeta1 is its' ability to stimulate *Tgfbeta* gene expression (Flanders et al., 1995; Van Obberghen-Schilling et al., 1988). In VPs, the regulation of *Tgfbeta* transcripts by TGFbeta1 was observed over a seven-hour culture but not over six days (Table 7.2). This suggests that the effect of TGFbeta1 on *Tgfbeta* transcript levels is only short term. No regulation of *Tgfbeta* transcript levels by TGFbeta1 was observed in the VMP, indicating epithelial-mesenchymal interactions might be required for the changes in *Tgfbeta* transcript levels observed in the VP.

Another observation is the difference in regulation of *Tgfbeta* gene expression by testosterone observed between the developing prostate in this study and the adult prostate previously studied (Bacher et al., 1993; Itoh et al., 1998a; Kyprianou and Isaacs, 1989). In the adult prostate *Tgfbeta* transcript levels increased after castration and the increase in *Tgfbeta* transcript levels could be repressed by androgens. The rapid changes in *Tgfbeta* transcript levels suggested that testosterone may have regulated *Tgfbeta* transcription and did not just cause a change in the ratio of number of cells expressing *Tgfbeta* transcripts. In addition, Itoh *et al.* (1998) demonstrated that *Tgfbeta* transcript levels were not regulated in isolated stromal or epithelial cells by testosterone, suggesting that the regulation of *Tgfbeta* transcript levels by testosterone requires stromal-epithelial interactions. In our experiments testosterone did not repress *Tgfbeta* transcript levels in the VP suggesting that *Tgfbeta* transcript levels are regulated using different mechanisms in developing compared to adult VPs. Also this suggests that mesenchymal-epithelial interactions observed in the developing prostate are different to stromal-epithelial interactions in the adult prostate. However, interestingly, *Tgfbeta1* and *Tgfbeta3* transcript levels were repressed in the VMP, which was similar to changes observed in the adult VP, suggesting that certain developmental mechanisms of regulation are maintained in adulthood.

Previous data has shown that complex interactions between testosterone and TGFbeta signaling may exist (Chipuk et al., 2002; Hayes et al., 2001; Kang et al., 2001), and these interactions will be discussed in context to data observed in this thesis. TGFbeta1 downstream signaling factor, Smad3, has been demonstrated to bind to AR and inhibit AR-mediated transcription (Hayes et al., 2001). The inhibition of AR-mediated transcriptional changes was demonstrated in our system as TGFbeta1 inhibited the effect of testosterone in the VMP. Also Smad3 has been shown to function as a co-regulator to enhance AR-mediated transactivation (Kang et al., 2001). Again this may be relevant to the VMP as *Tgfbeta2* transcripts levels increased only in the presence of testosterone and TGFbeta1. AR has also been shown to repress TGFbeta signaling (Chipuk et al., 2002) and this effect may be apparent in the VP in regard to some genes.

It has previously been demonstrated that *Tgfbeta1* and *Tgfbeta3* (but not *Tgfbeta2*) were regulated by the Sp1 transcription factor, indicating differential transcriptional regulation of the *Tgfbeta* genes (Geiser et al., 1993). Differential regulation of *Tgfbeta* genes was observed in both cultured VMPs and VPs. As the Sp1 transcription factor has already been shown to regulate *Tgfbeta1* and *Tgfbeta3* this suggests that Sp1 may be involved in the regulation of the *Tgfbeta* genes in the VMP and VP. Hence it could be suggested that the transcription factor Sp1 may be involved in regulating *Tgfbeta* transcript levels in prostate development. In fact the regulation of *Fgf10* transcript levels by TGFbeta1 was shown to involve the Sp1 transcription factor binding site in the *Fgf10* promoter and Sp1 has been shown to be involved in TGFbeta signaling pathways (Brodin et al., 2000; Feng et al., 2000b; Li et al., 1998). This suggests that complex pathways may be regulating the growth and development of the VP that involved the Sp1 transcription factor.

The difference in *Tgfbeta* transcript regulation between male and female prostatic rudiments becomes more apparent when comparing the regulation of *Tgfbeta* transcript levels in the VP to the VMP+UR using Table 7.2 and 7.3. The major difference between the VMP and VP is the budding and branching of epithelial and differentiation of stroma

in the VP. This suggests that epithelial/stromal interactions are regulating or are required for TGFbeta1 and testosterone regulation of *Tgfbeta* transcript levels. These observations also suggest that the *in vivo* effects of androgens and TGFbeta1 on *Tgfbeta* gene expression are regulated indirectly through a complex networks of growth factor signaling pathways between stroma and epithelial cells, and that differences occur in gene regulatory pathways between male and female and developmental and adult prostatic rudiments.

In conclusion it has been shown that TGFbeta1 regulates VP growth and branching morphogenesis and that an increase in epithelial bud number is not a result of increased proliferation of epithelial cells. It has also been demonstrated that TGFbeta1 has differing effects on stromal and epithelial cell proliferation in the proximal compared to distal regions to urethra in the VP. TGFbeta1 and testosterone regulated *Tgfbeta1* and *Tgfbeta3* transcript levels differently than *Tgfbeta2* transcript levels, indicating differential regulation of *Tgfbeta* isoforms. As a previous report has shown that testosterone and TGFbeta1 do not regulate *Tgfbeta* transcript levels in isolated prostate stromal and epithelial cells (Itoh et al., 1998a) it can be suggested that the regulation of transcripts in prostatic rudiments requires or is due to mesenchymal-epithelial interactions. Hence the regulation of *Tgfbeta* transcript levels in prostatic rudiments may involve complex networks of epithelial-mesenchymal interactions. Also it was demonstrated that testosterone repressed TGFbeta1 transcriptional changes in the VP, and TGFbeta1 inhibited testosterone transcriptional responses in the VMP. However testosterone did not inhibit the changes induced by TGFbeta1 in the growth of the VP suggesting that the regulation of *Tgfbeta* transcripts by testosterone and TGFbeta1 are not the main causative agents observed in changes in VP phenotype.

8 Discussion

The aim of this thesis was to investigate the regulation of *Fgf10* gene expression in prostatic development. A primary stromal cell system from the VMP (primary VMPC) was established and characterised and proposed a suitable *in vitro* model for these studies. Hence, the regulation of *Fgf10* transcript levels by TGFbeta1 and testosterone was initially investigated in primary VMPC. As TGFbeta1 repressed *Fgf10* transcript levels in primary VMPC, these studies were extended into organ cultures of VMP and UR, and VP. Primary VMPC were also used to analyse the *Fgf10* promoter to identify regions that regulated *Fgf10* gene expression and any regions of the promoter that mediated TGFbeta1 action. Owing to the regulation of *Fgf10* transcript levels by TGFbeta1 and the effect of TGFbeta1 on VP growth (Itoh et al., 1998a), FGF10 and TGFbeta1 appear to play opposing roles in prostate development. The function of TGFbeta1 in prostate development, and the regulation of *Tgfbeta* transcript levels by testosterone and TGFbeta1 were addressed in organ cultures of VP and VMP. The regulation of *Fgf10* and *Tgfbeta* gene expression by TGFbeta1 and testosterone proved complex, possibly involving interactions between epithelial and stromal cells.

8.1 Primary VMPC

Prostate organogenesis involves numerous interactions between epithelial and mesenchymal cells (Chung et al., 1991; Cunha et al., 1983; Tenniswood, 1986). These interactions induce ductal budding and branching morphogenesis of the prostate. Investigation of these poorly understood developmental processes is important, as inappropriate ductal branching occurs in prostatic disease (McNeal, 1988). Prostatic cell lines have been used to investigate the regulation of factors thought to be involved in prostate development and disease (Gerdes et al., 1998; Itoh et al., 1998a; Karan et al., 2002; Wilding et al., 1989). However the majority of research has focused on cell lines

derived from the epithelium, as most tumours arise in glandular epithelial cells (Gmyrek et al., 2001; Peehl and Stamey, 1986; Tang et al., 1999). However the inductive component of prostate budding and development is the mesenchyme/stromal tissue (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980; Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985). As a result, recent research has focused on prostate stromal cells as an important component in regulation of epithelial mitogenesis and differentiation in prostate cancer (Hall et al., 2002; Tuxhorn et al., 2002a; Tuxhorn et al., 2002b). One factor expressed in neonatal stromal tissue and known to regulate prostate growth and epithelial proliferation is FGF10 (Thomson and Cunha, 1999). FGF10 is therefore an important factor involved in prostate development and it may also be involved in prostate cancer. The aim of this thesis was to examine regulation of *Fgf10* gene expression during prostate development. Hence for these studies, a stromal cell type that expressed *Fgf10* and that was derived from the inductive tissue was required. Stromal cells in the VP have been shown to express *Fgf10* transcripts (Thomson and Cunha, 1999) and therefore the VP is an obvious candidate for disassociation into primary stromal cells. However, the rat male urogenital structures cannot be visibly subdivided into a bladder and a definite urogenital sinus until e17 (Hayward et al., 1996a). It would be desirable not to include non-inductive tissue in the dissection, and accurate dissection of the VP without including non-VP urogenital sinus is difficult until distinct VP structures are visible in the rat at e19. However by e19 solid cords of epithelium have been seen growing from the UGE (Hayward et al., 1996a). The removal of the epithelial buds from VPs to obtain a cell suspension of only stromal cells would be difficult owing the size of the organ. Also, epithelial budding is associated with stromal cell differentiation in the VP (Hayward et al., 1996b) and in this thesis it was demonstrated that the presence of epithelium may effect the regulation of *Fgf10*. Thus VP tissue was deemed unsuitable, as a cell type was required that had not been subjected to differentiation in response to epithelium.

The VP develops from UGE budding into a discrete pad of mesenchyme, called the VMP. A female VMP has been identified, and prostatic budding can be induced in the

female VMP in the presence of testosterone (Lasnitzki and Mizuno, 1977; Takeda et al., 1986; Timms et al., 1995). *Fgf10* transcripts have been localised to both the male and the female VMP by *in situ* hybridisation (Thomson and Cunha, 1999) and RNase protection (Fig. 3.11). Therefore, the VMP is made up of a cell type that expresses *Fgf10*, has not differentiated in response to epithelium and is capable of inducing prostate-like budding. Primary cultures of VMPC were tested to see if these cells were a good model for studying endogenous *Fgf10* gene regulation.

In order to validate the use of primary VMPC in studies of *Fgf10* gene regulation and to compare *Fgf10* promoter construct activity in *Fgf10* expressing cells to non-*Fgf10* expressing cells, primary VMPC were compared to primary cells derived from non-*Fgf10* expressing tissue that cannot induce prostate development. It is known that non-VMP URS (dissected from the caudal region of the urethra) is not capable of inducing prostate organogenesis (Timms et al., 1995), and does not express *Fgf10* (Thomson and Cunha, 1999). Therefore primary VMPC were compared to primary URSC.

The P0 female VMP is relatively small and in close contact with UR, and a smooth muscle layer separates the VMP from the UR (Thomson et al., 2002). The VMP is SM-negative *in vivo* but the micro-dissected VMP included some of the smooth muscle layer from the UR (shown by western blotting in figure 3.9). Analysis of primary VMPC revealed that all cells expressed SM α actin, a smooth muscle marker. This suggested either that only the cells that expressed SM α actin plated out or that non-SM α actin expressing cells expressed SM α actin upon explant. The dissected VMP would have consisted of a heterogeneous population of mesenchymal and SM α actin cells. It was more likely that mesenchymal cells began to express SM α actin on disassociation and plating out, as the majority of the cells in the micro-dissected tissue would have been from the VMP. However, to further confirm that primary VMPC are of VMP origin a comparison of primary VMPC and VMP was performed. Primary VMPC were also compared to primary URSC and UR as a comparison to non-VMP derived cells and tissue.

A key difference between primary VMPC and URSC is the rate of cell senescence. In past literature it has been suggested that the number of cell divisions *in vitro* is directly associated with the number of cell divisions *in vivo* (Hayflick, 1965; Martin et al., 1970). As the VMP eventually disappears after about P12 in the female it could be suggested that the number of cell divisions in a female VMP would be considerably less than cells in the URS. Hence the number of cell divisions in culture would be lower in primary VMPC than URSC, which is reflected in cells in culture. There could be other reasons for the lower number of cell divisions such as the loss of a VMP survival factor on explant. Overall, it is likely that the main source of primary VMPC were VMP cells and not SM cells.

Another key difference between primary VMPC and URSC is the expression pattern of *Tgfbeta* transcripts. Previous research has shown that *Tgfbeta* transcripts are expressed in cells derived from prostate tissue (Itoh et al., 1998a; Story et al., 1996), and through inhibiting the growth of both epithelial and stromal cells TGFbeta has been proposed as having dramatic effects on the prostate (Ilio et al., 1995; Martikainen et al., 1990; Story et al., 1993; Sutkowski et al., 1992). Differential transcriptional regulation of *Tgfbeta* genes has been previously shown (Geiser et al., 1993), and targeted disruption of *Tgfbeta* genes result in different phenotypes (reviewed in (Dunker and Krieglstein, 2000)), suggesting that TGFbeta isoforms may have different roles during development. Our results demonstrated that primary VMPC and URSC expressed different levels of *Tgfbeta1*, *Tgfbeta2* and *Tgfbeta3* transcripts, and the transcript levels were similar to levels expressed in the organ rudiments from which they were derived. Thus we believe our primary cells are a good reflection of the *in vivo* rudiments. This also suggested that the different *Tgfbeta* isoforms could have different roles in development of the female UGS to the female VMP e.g. as *Tgfbeta3* was more highly expressed in the VMP than the UR (Fig. 3.10) thus *Tgfbeta3* may be involved in the formation of the VMP. The difference in *Tgfbeta* isoform expression between primary VMPC and URSC also suggested that primary VMPC were derived from the VMP and not UR.

The idea of comparing primary VMPC to primary URSC was mainly based on the fact that the URS was shown not to express *Fgf10* transcripts by *in situ* hybridisation (Thomson and Cunha, 1999). However, RNase protection assay revealed that low levels of *Fgf10* transcripts were expressed in the URS. *Fgf10* transcripts were also expressed in primary URSC at similar levels to primary VMPC (Fig. 3.11). The elevated expression of *Fgf10* transcripts in primary URSC may be a wound response to the cells being disassociated from the URS, as *Fgf10* may be a primary factor involved in the process of wound healing (Tagashira et al., 1997). However, some reports suggest that FGF10 is not involved in wound healing (Beer et al., 1997). The levels of *Fgf10* transcripts in the wounding process decreased after the initial increase after injury (Tagashira et al., 1997), and this was reflected in cells in culture. The levels of *Fgf10* transcripts in primary URSC decreased after the second passage while levels of *Fgf10* in primary VMPC did not change with passage number. This further suggested that the increase in *Fgf10* transcript levels in primary URSC was a response to disassociation of the URS, while the *Fgf10* transcripts were expressed in primary VMPC because of the expression already observed in the VMP rudiment. Also it was impossible to tell which cells in the URS expressed *Fgf10*, and whether the *Fgf10* expressing cells were the only cells to survive on plating out or if these cells were overgrowing the other cells. Another possible explanation for the increase in *Fgf10* transcripts levels may be that factors produced in the epithelium are regulating its expression. As the URS is in close contact with URE, factors produced by the URE may have repressed *Fgf10* transcript expression in the URS. When URS cells were disassociated and plated out they were no longer in contact with URE cells, therefore the repression on *Fgf10* transcript levels by URE no longer occurred hence expression of *Fgf10* transcripts was increased. Recently, regulation of *Fgf10* transcript levels was investigated in the lung (Bellusci et al., 1997b) and *Fgf10* transcript levels were repressed by factors produced in the epithelium, which correlates with the hypothesis described above for the prostate. Also in lung studies it was observed that in the absence of epithelium *in vitro*, mesenchymal cells have a marked increase in the *Fgf10* transcript levels (Lebeche et al., 1999). It was speculated

that epithelial cells in the intact lung secrete factors that are inhibitory for *Fgf10*. Collectively this data suggests that *Fgf10* gene expression in the lung could be regulated by factors produced in the epithelium and as factors controlling branching morphogenesis in the lung and prostate have proved to be similar this mechanism may be carried through to regulation of factors in the prostate. Also as epithelia may repress *Fgf10* transcript levels you would expect more *Fgf10* transcripts in the P0 VMP compared to the P0 VP. In fact this was demonstrated in Fig. 3.11; the VMP expressed four times more *Fgf10* transcripts than the VP.

Primary URSC were meant to act as a negative control for promoter analysis i.e. cells that do not express *Fgf10*. The fact that primary URSC expressed *Fgf10* transcripts raised the question of whether primary URSC could have been used in experiments addressing the regulation of *Fgf10* gene expression as well as primary VMPC. However, *Fgf10* gene expression decreases in primary URSC after the second passage. This suggested that primary URSC could be used at passage three in the promoter analysis (i.e. when the cells did not express *Fgf10* transcripts), but at this passage the URSC were hard to transfect. The decrease in *Fgf10* transcript levels after the second passage in primary URSC also suggested that *Fgf10* gene expression might have been regulated by different factors to *Fgf10* gene expression in primary VMPC. Also through the characterisation of primary VMPC and comparing them to primary URSC it became apparent that the two cell types had key differences that were characteristic to the rudiment from which they were derived. Therefore primary URSC may prove to be a useful cell type to examine and compare *Fgf10* promoter construct activity at early passage dates, although whether this would say anything about regulation of *Fgf10* in the prostate is unclear.

In conclusion primary VMPC were a good, but not perfect, model to examine the regulation of *Fgf10* transcript levels because 1) they express *Fgf10* transcripts, 2) the number of cell divisions may be representative of the life span of the VMP, 3) the *Tgfbeta* transcripts expression pattern was similar to that of the VMP, 4) they expressed

AR (described in chapter 3), and 5) they represent a cell type derived from a pad of mesenchyme that can induce prostate organogenesis. However as primary VMPC started to senesce after passage number one it was concluded that any experiments would be performed before or at this passage number.

8.2 Regulation of *Fgf10* Transcript Levels

Experiments were performed to determine whether testosterone and/or TGF β 1 regulated *Fgf10* transcript levels in cells and prostatic organ rudiments.

8.2.1 The Effect of Testosterone

Previous studies have shown that androgen/AR action in mesenchymal cells is essential for prostate development (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980; Shannon and Cunha, 1983; Takeda and Mizuno, 1984; Takeda et al., 1985). Many studies have attempted to identify paracrine factors that are stimulated by testosterone and that promote prostate development (Finch et al., 1995a; Lu et al., 1999; Peehl and Rubin, 1995; Sugimura et al., 1996; Yan et al., 1992). FGF10 has been shown to function as a mesenchymal paracrine regulator of epithelial growth and branching morphogenesis in the VP (Thomson and Cunha, 1999). *Fgf10* gene expression has been shown to be regulated by testosterone in cells *in vitro* and has been proposed to be a mediator of androgen action (Lu et al., 1999). However, there is some controversy concerning the regulation of *Fgf10* gene expression by testosterone (Thomson, 2001), hence it was decided to further investigate this in our culture system. Data presented here suggested that testosterone did not regulate *Fgf10* transcript levels in primary VMPC cultured in the presence of testosterone for either 7 or 48 hours. Also, culturing VPs in the presence of testosterone for either three or six days did not regulate *Fgf10* transcript levels. Taken together these results confirmed data published by Thomson and Cunha

(Thomson and Cunha, 1999). However, an increase in *Fgf10* transcript levels in response to testosterone was observed in NIH3T3 cells and in the female UGS.

Fgf10 transcript levels increased two-fold in NIH3T3 cells cultured for 48 hours in the presence of testosterone. This increase was only observed in the presence of FCS suggesting that testosterone was acting in conjunction with a factor within the serum. NIH3T3 cells lack AR shown by immunohistochemistry and western blotting (Fig. 4.1A,B); therefore the change in *Fgf10* transcript levels caused by testosterone must have occurred through an AR independent pathway. One such pathway may involve the human sex hormone-binding globulin (SHBG). It has been shown that biologically active steroids activated receptor-bound SHBG to cause a response in LNCaP cells, demonstrating a mode of action for steroid hormones that did not require interaction with a steroid receptor (Nakhla et al., 1990). This study demonstrated that when LNCaP cells were prebound with SHBG, addition of DHT resulted in a dose dependent increase in intracellular cAMP. SHBG globulin in the absence of DHT or DHT in the absence of SHBG was without effect. More recently it has been demonstrated that the ability of oestradiol to induce cAMP in breast cancer cells (MCF-7) is not due to the ligand-bound oestrogen receptor, but rather it was mediated via FCS (Fortunati et al., 1996; Fortunati et al., 1999). In addition it was shown that SHBG triggers the cAMP pathway in MCF-7 cells at physiologic concentrations. The evidence points to a similar effect observed in NIH3T3 cells. However, this was not further investigated in this thesis, as the effect did not occur in primary VMPC that represented a developmental prostate cell type.

As mentioned previously an increase in *Fgf10* transcript levels was also observed in the female UGS cultured with testosterone. *Fgf10* transcript levels increased in both the VMP+UR and UR rudiments of the female UGS, when cultured in the presence of testosterone for three days. It was proposed that *Fgf10* gene expression might not be directly regulated by androgens, and the observed elevated expression could be a result of androgens stimulating proliferation of *Fgf10* expressing cells. This would explain the increase in *Fgf10* transcript levels in both the VMP+UR and UR. Also this is the likely

explanation owing to the fact that an increase in VMP size (i.e. in cells that express *Fgf10*) was obvious in UGSs cultured with androgens after three days (personal communication S.Freestone). Other explanations for the change in *Fgf10* transcript levels involve the differentiation of cells in the female UGS. Testosterone may be regulating cellular differentiation that may result in cells that express a higher level of *Fgf10* transcripts. Testosterone has also been shown to affect the differentiation of SM cells in the female UGS (Thomson et al., 2002). In cultures of female UGS androgens regulated the thickness of the SM layer resulting in a 2.4-fold reduction in thickness. It is possible that *Fgf10* positive mesenchyme may differentiate into SM, but the presence of testosterone prevents this differentiation therefore there is more mesenchyme that expresses *Fgf10* transcripts. These processes propose an indirect pathway that androgens may regulate *Fgf10* transcript levels in the female UGS.

8.2.2 The Effect of TGFbeta1

The TGFbeta peptides are members of a large superfamily of highly conserved cytokines (Massague, 1998) that have been implicated in prostate development (Itoh et al., 1998a; Timme et al., 1994). Analysis of *Tgfbeta* transcript levels between e17.5 and P6 suggested that TGFbeta-signalling might participate in early VP development. These results agreed with previous data suggesting *Tgfbeta* isoforms are associated with fetal UGS tissues and prostate morphogenesis (Timme et al., 1994). A previous report suggested *Tgfbeta1* expression remains constant, and *Tgfbeta2* and *Tgfbeta3* expression are inversely related during prostate development (Itoh et al., 1998a). This report examined *Tgfbeta* expression patterns at P1, P20, P60 and P100 and compared values to P20. My studies examined levels during the neonatal period, when the complex processes of branching had relatively just commenced (Sugimura et al., 1986b), therefore providing more complete picture of how *Tgfbeta* isoform expression might correlate with early prostate development. Levels of *Tgfbeta1* and *Tgfbeta3* transcripts were increased and decreased at similar stages throughout prostate development, while *Tgfbeta2* did not follow the same expression pattern. Differential transcriptional

regulation of *Tgfbeta* genes has previously been shown by promoter analysis (Geiser et al., 1993). Our results suggest that the regulation of *Tgfbeta1* and *Tgfbeta3* gene expression was regulated differently to *Tgfbeta2* gene expression during prostate development.

The expression pattern of *Tgfbeta* isoforms suggested that TGFbeta plays an important role in prostate development. TGFbeta has been shown to inhibit prostate growth (Itoh et al., 1998a) playing an opposing role to FGF10, which has been shown to promote growth and development (Thomson and Cunha, 1999). As TGFbeta1 repressed *Fgf10* transcript levels in non-prostate cell lines (Beer et al., 1997; Lebeche et al., 1999), it was decided to investigate whether TGFbeta1 regulated *Fgf10* transcript levels in the prostate. Data presented here showed that *Fgf10* transcripts levels were reduced by fourteen-fold in primary VMPC cultured in the presence of TGFbeta1 for three hours. This repression was reversible, and after 48 hours *Fgf10* transcript levels had reverted back to normal levels. Repression of *Fgf10* transcript levels was also observed in NIH3T3 cells but this effect was less reversible. In that regard the result in NIH3T3 cells was different to what Beer *et al.* demonstrated, as their experiments showed that *Fgf10* transcript levels started to recover after eight hours in culture with TGFbeta (Beer et al., 1997). This may simply be a result of using different cell lines. The reversible effect of TGFbeta1 on *Fgf10* gene expression in primary VMPC might have involved complex interactions of TGFbeta-signalling pathways (discussed in chapter 1). The reversible effect of TGFbeta on *Fgf10* transcript levels observed in primary VMPC may have been caused by an increase in inhibitory SMAD expression resulting in negative feedback signalling that prevents TGFbeta1 action. Also the regulation of *Fgf10* transcript levels might not be a direct effect of SMAD induction but may be mediated via other TGFbeta-inducible genes. One such gene is TGFbeta-inducible early gene (TIEG). TIEG transcript levels increase rapidly after TGFbeta treatment but decrease after 4 hours (Cook et al., 1998; Subramaniam et al., 1995). Also TIEG has been shown to inhibit gene transcription (Cook et al., 1998; Yajima et al., 1997). The pattern of TIEG expression after TGFbeta treatment is highly suggestive that TIEG may repress

Fgf10 gene transcription when initially induced but when levels of TIEG drop less repression occurs and *Fgf10* transcript levels may return to normal. Future studies could examine the induction and kinetics of TIEG and SMAD expression in primary VMPC to determine which correlates with *Fgf10* transcript changes.

As TGFbeta1 negatively regulated *Fgf10* transcript levels in primary VMPC, we next extended our analysis to VMP rudiments grown *in vitro*. A similar regulation of *Fgf10* transcript levels by TGFbeta1 was observed in VMP organ rudiments, but not in UR organ rudiments. *Fgf10* transcript levels were decreased a maximum of three-fold in the VMP over seven hours which was comparable to the regulation of *Fgf10* transcript levels in primary VMPC at the same time point. The regulation of *Fgf10* transcript levels by TGFbeta1 in the VMP was not investigated at three hours as this length of time was postulated as not being long enough for TGFbeta1 to penetrate and affect *Fgf10* gene expression.

The presence of epithelium may inhibit the regulation of *Fgf10* transcript levels by TGFbeta1, as epithelial cells have been proposed to produce factors that regulate *Fgf10* transcript levels in the mesenchyme (Bellusci et al., 1997b). If this hypothesis is true, then no or very little repression of *Fgf10* transcript levels would be observed in the VP depending on the extent of epithelial budding. In fact the highest level of repression of *Fgf10* transcript levels by TGFbeta1 in the VP was observed on day one of culture, when epithelial branching was relatively low, and this repression was only half the level observed in the VMP. As the extent of epithelial branching increased, even less TGFbeta1-mediated repression of *Fgf10* transcript levels occurred. This data is highly suggestive that the epithelium is inhibiting the repression of *Fgf10* transcript levels by TGFbeta1 in the VP. This theory also may explain why TGFbeta1 did not regulate *Fgf10* transcript levels in the UR, as URS is in close contact with URE. If there are more buds then regulation of *Fgf10* may be more tightly controlled. Also the effect of TGFbeta1 on *Fgf10* transcript levels is reduced as VPs develop suggesting that the effect is temporal i.e. as the VP develops the effect of TGFbeta1 on *Fgf10* transcript levels is

progressively reduced. This hypothesis is substantiated as *Fgf10* may play a dual role in rat prostate development like branchless (*bnl*), an *Fgf* homologue, may play during *Drosophila* tracheal branch formation. *Bnl* regulates tracheal branching in two ways, by guiding tracheal cell migration and then inducing later programs of tracheal branching at the ends of growing primary branches (Sutherland et al., 1996). *Fgf10* may play a similar role in rat prostate development, by inducing bud outgrowth and later promoting and guiding branching morphogenesis. Hence it is possible that different molecules regulate *Fgf10* during these different stages of development, and TGFbeta1 may repress *Fgf10* transcript levels during early bud outgrowth and not during branching morphogenesis. This may explain why TGFbeta1 only regulates *Fgf10* transcripts during early prostate development in the VP.

Androgens promote the growth and development of the prostate and TGFbeta1 negatively regulates this process, suggesting that interactions between androgen and TGFbeta signalling pathways may play significant roles during the development of the prostate. Recent reports have identified Smad3 as an AR coregulator, and that this interaction may either enhance (Kang et al., 2001) or inhibit (Hayes et al., 2001) AR-mediated transcription. Also AR has been shown to repress TGFbeta signalling through interactions with Smad3 (Chipuk et al., 2002), suggesting that androgens can inhibit TGFbeta-mediated transcription. TGFbeta1 was shown to repress *Fgf10* transcript levels in primary VMPC, and the addition of androgens inhibited this effect, indicating that androgens can inhibit TGFbeta-mediated transcription in prostate stromal cells. This suggests that complex interactions between androgen and TGFbeta-signalling may regulate prostate development.

8.2.3 *Fgf10* Promoter Analysis

Promoter analysis is a useful tool for the identification of factors involved in regulating genes implicated in prostate development (Bloom et al., 1996; Fasciana et al., 1996; Fenton et al., 1996; Finch et al., 1995b). Hence, a method of addressing the regulation

of *Fgf10* gene expression was to analyse the *Fgf10* promoter. The first stage of characterising the *Fgf10* promoter was to map the transcription start site. 6 kb of genomic sequence 5' to the translation start site was searched using NIX analysis for any putative TATA-containing promoters. No characteristic TATA-promoters were identified, so genomic sequences that were 5' to the *Fgf10* translation start site (mouse and human), and a cDNA sequence (sheep) for *Fgf10* were compared using VISTA analysis. A comparison between genomic sequences was performed as the percentage identity between sequences might fall 5' to the transcription start site, i.e. in non-transcribed sequences, and between genomic and cDNA, as the 5' end of cDNA should lie on a transcription start site. In fact, the sequence identity fell in the same promoter region (approximately 1300 nts 5' of the translation start site) in the comparison between mouse and human, and mouse and sheep. Hence, this region might contain a putative transcription start site. Riboprobes were designed from -1438 to -981 (probe1), and from -981 to -469 (probe2) (5' of the translation start site), to map transcription start sites. RNase-digested bands were observed using both riboprobes and no fully protected band was observed using probe2, therefore *Fgf10* transcription was unlikely to start between -1438 and -981, and more likely to start 3' of this region. However to verify that the RNase-digested bands observed in probe1 were not transcription start sites a further riboprobe (probe3) was cloned spanning probe1 and probe2, from -1131 to -631. An RNase-digested band of approximately 70 nts in length was observed which was highly suggestive that the RNase-digested bands detected using probe2 (between -700 and -770) represented a transcription start site. Probe4 (from -841 to -427) and probe5 (from -841 to -500) were cloned to exactly identify which nucleotide represented the transcription start site, and probe6 was cloned from sequence 3' to the transcription start site as a control riboprobe that should be fully protected. Numerous protected bands were observed using probe4 and probe5 suggesting that there was more than one transcription start site for *Fgf10*. Amongst these bands, a single band was consistently stronger than others with every probe used; this probably represented the major transcription start site for *Fgf10*.

Analysis of the *Fgf10* promoter revealed that it is a TATA-less promoter. Many promoters do not contain consensus TATA boxes and TATA-less promoters can either retain the ability to direct transcription initiation from a specific nucleotide or direct transcription initiation through multiple sites (Smale, 1997). The *Fgf10* promoter seems to direct transcription initiation through multiple sites. No TATA box seemed to be present upstream of this site. It has been shown that TATA-lacking promoters may contain an element called an initiator (Inr) and the Inr can overlap the transcription start site of both TATA-containing and TATA-lacking promoters (Smale, 1997). The first functionally derived consensus sequence for Inr activity was derived from a mutant analysis of over 80 generated Inr (Javahery et al., 1994). A loose consensus sequence of (C/T)(C/T)A⁺N(T/A)(C/T)(C/T) was revealed. The Inr of the *Fgf10* promoter (GGCTTCA) was not similar to the published consensus sequences, however other typical features of TATA-less promoters were identified.

Further promoter analysis revealed a GC-rich region (63 %), including Sp1 consensus sites in the vicinity of the transcription initiation site. A similar region was also identified in the human promoter, with a GC-rich region (64 %) in the vicinity of the transcription start site, however an identical Sp1 consensus site was not found. These features are typical of TATA-less promoters with multiple transcription initiation sites (Pugh and Tjian, 1990). Also the GC-rich region was reminiscent of CpG islands. CpG islands can be 0.5-5 kilobases in size with a characteristically high G+C content (60-70 %). Initially CpG islands were identified as short stretches of DNA that contained a nucleosome-free region allowing for easy access for transcription factors (Bird, 1986). Approximately half of all mouse genes, mainly house keeping genes, and about 40 % of genes with a tissue-restricted pattern (i.e. *Fgf10*) contain CpG islands (Antequera and Bird, 1993). As CpG islands allow for easy access to transcription factors, the putative CpG island in the *Fgf10* promoter could possibly harbour sites responsible for regulation of *Fgf10* gene expression. This region was confirmed as being a putative CpG island using NIX analysis.

Another characteristic of TATA-less promoters is the presence of a downstream promoter element (DPE). Burke and Kadonaga (1996) identified a conserved element about 30 bp downstream of the transcription start sites in a large number of *Drosophila* genes. A consensus sequence for the DPE was suggested to be (A/G)G(A/T)CGTG (Burke and Kadonaga, 1996). A similar sequence was identified (GGACTTG) in the *Fgf10* promoter exactly 30 bp downstream of the transcription start site. Overall although the Inr consensus sequence does not exactly match the *Fgf10* transcription start site sequence, other characteristic features of the promoter suggest that this site is most probably the major transcription start site.

Many regions of the *Fgf10* promoter were highly conserved between mouse and human, and these conserved regions 5' to the transcription start site might contain regulatory elements. The conserved regions include the area containing the putative CpG island, and in conjunction with Dr. Justin Grindley these regions were characterised. The conserved regions were characterised by two methods, by cloning them into an enhancer vector and by deleting the regions from the *Fgf10* promoter. Two regions (A and F) of the *Fgf10* promoter were shown to regulate the activity of SV40 enhancer vector, but when these regions were deleted from the *Fgf10* promoter sequence in pA3Lucm no change in promoter activity was observed. This suggests these regions have no direct effect on *Fgf10* transcription in the context of the *Fgf10* promoter. In contrast region G (which contained the putative CpG island) had no effect on the SV40 enhancer vector, but when deleted from the *Fgf10* promoter *Fgf10* transcription was repressed. As this region was identified as being a putative CpG island capable of harbouring transcription factor binding sites, and when deleted from promoter constructs altered *Fgf10* transcription, it can be suggested that region G is highly likely to contain sites that regulate *Fgf10* gene expression. Region G was analysed using GeneJockey II and a putative Sp1 binding site was identified.

Certain genes have been shown to be Sp1-dependent and the Sp1 site involved participated in transcription initiation regardless of whether they contained TATA or

TATA-less promoters (Botella et al., 2001; Brodin et al., 2000; Datta et al., 2000; Feng et al., 2000a; Moustakas and Kardassis, 1998). Hence as *Fgf10* transcription was repressed when the Sp1 site (with surrounding regions) was deleted from the *Fgf10* promoter construct, it is highly likely that the Sp1 site was participating in *Fgf10* transcription. Also studies have shown that regulation of gene expression by TGFbeta involves interactions of the Sp1 transcription factor and Sp1 binding sites (Bloom et al., 1996; Botella et al., 2001; Brodin et al., 2000; Datta et al., 2000; Feng et al., 2000b; Geiser et al., 1993; Li et al., 1998; Li et al., 1995). As TGFbeta1 repressed *Fgf10* transcript levels in cells and organs it was highly likely that the consensus Sp1 site in region G might be involved in regulating *Fgf10* gene transcription. To test these hypotheses, *Fgf10* promoter constructs were transfected into primary VMPC and treated with TGFbeta1. The Sp1 site was deleted from the *Fgf10* promoter in two types of constructs to address the effect on promoter activity and TGFbeta1-mediated repression. Initially the Sp1 binding site was 5' deleted leaving all the 3' sequence in pA3Lucm and its activity was compared to the activity in promoter constructs containing the Sp1 site. However this might not address whether regions 5' and in close proximity to the putative Sp1 binding site regulated *Fgf10* gene expression. As the activity of *Fgf10* promoter constructs did not change up to ΔA-G, it was decided to delete the Sp1 site from ΔA-D. ΔA-D contained enough sequence 3' of the Sp1 site to harbour possible areas involved in *Fgf10* regulation and it was easier to clone this region than the Sp1 site deleted from the full length construct. The Sp1 site seemed to mediate TGFbeta1 repression of *Fgf10* gene expression to levels observed in the core promoter. However only a two-fold reduction in activity was observed in the presence of TGFbeta1 but TGFbeta1 repressed *Fgf10* transcript levels by up to fourteen-fold in primary VMPC. Although there was only a two-fold decrease in levels, these levels were the same as levels observed in constructs containing only the core promoter suggesting that TGFbeta1 inhibited the majority, if not all, of *Fgf10*'s promoter activity, but not basal activity. The difference between the regulation of promoter activity and endogenous *Fgf10* transcript levels by TGFbeta1 was probably due to the limitations of the

transfection. Also binding sites that may mediate TGFbeta1 action could be found in other parts of the *Fgf10* promoter that were not studied in this thesis.

The deletion of the Sp1 site from ΔA-D resulted in a reduction in basal activity of the *Fgf10* promoter, again to similar levels observed in the core promoter, suggesting that the Sp1 site was also involved in mediating basal transcriptional activity. In construct -Sp1, i.e. the Sp1 site was 5' deleted, the activity was reduced to a lesser degree than observed in ΔA-DΔSp1, raising the question of whether *Fgf10* gene expression was mediated through the Sp1 site. However ΔSp1 only represents a small part of the promoter while ΔA-DΔSp1 represents a larger proportion of the promoter, leading to the conclusion that the effect observed in ΔA-DΔSp1 was probably a more accurate assessment of the function of the Sp1 site. It is not known why there was a difference between ΔSp1 and ΔA-DΔSp1 activity in transfected cells but it may be a result of cryptic sites in the plasmid that may have altered the regulation of transcription in the ΔSp1 plasmid construct. However this explanation is difficult to justify as the same vector was used for each promoter construct and therefore they contained the same potential 'cryptic sites'.

In conclusion we have identified a promoter whose basal transcriptional activity is regulated by Sp1. Numerous other studies have identified similar regions of promoters that regulate transcriptional activity (Botella et al., 2001; Brodin et al., 2000; Datta et al., 2000; Feng et al., 2000a; Moustakas and Kardassis, 1998), however the majority of these studies demonstrated that TGFbeta signalling induced transcription while our results showed that TGFbeta inhibited *Fgf10* transcription. This may be due to the increase in TIEG proteins (briefly described in chapter 1). TIEGs are induced by presence of TGFbeta (Cook et al., 1998; Subramaniam et al., 1995) and may cause a repression of gene transcription (Tachibana et al., 1997; Tau et al., 1998; Yajima et al., 1997). TIEGs also have the ability to bind to GC-rich regions, like Sp1 sites (Cook et al., 1999; Cook et al., 1998; Yajima et al., 1997) therefore they may mediate their effect via competing for binding with Sp1. By competing for binding and thus preventing Sp1 mediated

transcription, TIEG may reduce *Fgf10* transcript levels. The regulation of *Fgf10* transcript levels by TGFbeta1 is transient and *Fgf10* transcript levels begin to return to normal levels after seven hours in culture (chapter 4). However TIEG transcript levels increase rapidly in cells after treatment with TGFbeta and levels return to normal after 4 hours in culture (Cook et al., 1998; Subramaniam et al., 1995). The change in transcript levels of TIEG and *Fgf10* are therefore inversely related; as TIEG transcripts increase *Fgf10* transcripts decrease. This is suggestive that TGFbeta1 may be regulating *Fgf10* transcript levels via the induction of TIEG. This proposes a novel way in which TGFbeta1 may mediate *Fgf10* gene transcription and gene expression. However the expression and regulation of TIEG in primary VMPC and the binding of TIEG in competition to Sp1 to the *Fgf10* promoter needs to be further investigated to verify this hypothesis.

8.3 Regulation of VP Development by TGFbeta1

Numerous growth factors have been identified as mediators of mesenchymal-epithelial interactions in prostate development (Finch et al., 1995a; Peehl and Rubin, 1995; Thomson and Cunha, 1999; Thomson et al., 1997; Timme et al., 1994). TGFbeta has been proposed to be one such factor, and it has been shown that TGFbeta1 can inhibit prostate growth (Itoh et al., 1998b; Tanji et al., 1994). It has also been suggested that TGFbeta1 probably plays a significant role in the induction of prostatic budding (Timme et al., 1994). As FGF10 can promote prostate growth and is an important factor for prostate development (Thomson and Cunha, 1999), and TGFbeta1 can regulate *Fgf10* transcript levels (shown in chapters 2 and 3), it can be suggested that TGFbeta1 and FGF10 may play opposing roles in development of the prostate. So next the effect of TGFbeta1 on VP growth and development was investigated.

The role of TGFbeta1 in the growth and development of the neonatal VP was investigated using an organ culture system. TGFbeta1 was shown to reduce the area of VPs confirming TGFbeta1 as an inhibitor of prostate growth, but TGFbeta1 also

increased the number of epithelial buds around the periphery of the organs. This suggested that TGFbeta1 might have multiple roles in prostate growth and development. Inhibition of growth of the VP by TGFbeta has been previously demonstrated (Itoh et al., 1998a). Itoh *et al.* (1998) also demonstrated the ability of TGFbeta to inhibit ductal branching morphogenesis. However, in our study androgens and TGFbeta1 were found to promote the number of buds suggesting that TGFbeta1 might promote branching morphogenesis. Itoh *et al.* (1998) examined the number of branching ducts throughout the organs using a two-dimensional image, while our data determined the number of buds around the periphery of the organs. This method was used because it was decided that it was too difficult to determine the number of branching ducts, but counting the number of buds around the periphery of VPs was an accurate and easy way of comparing the effect of TGFbeta1 on branching morphogenesis. Hence, the different results may reflect the different methodologies.

It has previously been shown that the TGFbeta1-mediated transcriptional changes could be inhibited by testosterone in cell lines (Fig. 4.8) (Chipuk et al., 2002) and in organ culture (Fig. 7.7). In the current study the changes in growth induced by TGFbeta1 in the VP were not inhibited by testosterone, suggesting the effects caused by TGFbeta1 were not transcriptional responses that were regulated by this mechanism. This proposes that the transcriptional changes, described in this thesis, caused by TGFbeta1 and that were inhibited by testosterone were not fully responsible for the changes in growth induced by TGFbeta1.

To determine if the changes in size and bud number of VPs were due to TGFbeta1 regulating the proliferation rate of cells, proliferative index was calculated by BrdU incorporation. TGFbeta1 has been well documented as inhibiting cell proliferation (Blanchere et al., 2001; Bretland et al., 2001; Ilio et al., 1995; Wilding et al., 1989), but TGFbeta1 has also been shown to increase the proliferation rate of cells (Hsu et al., 1994; Kansra et al., 2000; Lamm et al., 1998; Schroy et al., 1990). The increase in proliferation rate by TGFbeta1 has been associated with undifferentiated cell types (Hsu

et al., 1994; Kansra et al., 2000; Lamm et al., 1998; Schroy et al., 1990), suggesting that the proliferation rate of undifferentiated cells may be stimulated by TGFbeta1 while the proliferation rate of more differentiated cells are inhibited by TGFbeta1. It was demonstrated that TGFbeta1 had a similar effect in cells at different stages of differentiation in the VP (Fig 7.6). TGFbeta1 increased the proliferation of relatively undifferentiated cells but inhibited proliferation of differentiated cells. Also testosterone is known to promote differentiation of prostate epithelial cells, and it was demonstrated that after six days the inhibition of proliferation in the proximal region by TGFbeta1 was greater in organs cultured with testosterone than those cultured without testosterone. Hence this result strongly suggests that TGFbeta1 inhibits the proliferation of differentiated cell types while promoting proliferation of undifferentiated cell types.

The changes in cell proliferation rate, in different regions of VPs cultured with TGFbeta1, also correlated with the change in their two-dimensional size. Although TGFbeta1 increased the proliferation rate of cells this was only a significant increase after six days in culture. In contrast the inhibition of cell proliferation in the proximal regions was apparent at all time points examined. This suggests that overall the organs cultured with TGFbeta1 should be smaller than those cultured with vehicle, which is representative of what was demonstrated. Also the increase in proliferation was only observed in the absence of testosterone. TGFbeta1 might not have increased the rate of proliferation in cells in the presence of testosterone as the maximum proliferation rate might have been reached in response to testosterone in this system. However this observation might explain why the difference in size between VPs cultured with TGFbeta1 was greater in VPs cultured in the presence of testosterone (32 % decrease) than those cultured in the absence of testosterone (21 % decrease).

It was also shown that bud number increased in VPs cultured with TGFbeta1 in the presence and absence of testosterone, but cell proliferation was only increased in the absence of testosterone. This suggests that an increase in bud number was not related to an increase in proliferation rate of epithelial cells, which agrees with previous data that

has shown that increased cell proliferation is not a prerequisite for budding in lung explants (Nogawa et al., 1998).

The regulation of *Tgfbeta* gene expression in the prostate has previously been investigated in adult prostates and prostate cells lines (Itoh et al., 1998a; Kyprianou and Isaacs, 1989), but none have addressed it during neonatal prostate development. The regulation of *Tgfbeta* transcript levels was investigated to determine if any changes in transcripts levels could be correlated to the changes observed in VP morphology. To investigate the regulation of *Tgfbeta* transcript levels male and female prostatic organ rudiments were cultured with TGFbeta1 and/or testosterone. However, the comparison between the changes in *Tgfbeta* transcript levels in the VMP and VP is difficult owing to the different lengths of time the organ rudiments were cultured, but Table 7.2 and 7.3 were designed for an easier comparison of results. We have demonstrated that both TGFbeta1 and testosterone can regulate the levels of *Tgfbeta* transcripts in cultured organ rudiments. However the pattern of gene regulation was different between male and female organ rudiments and on the length of time in culture. This suggests that epithelial interaction in the male rudiment may be affecting the regulation of *Tgfbeta* transcripts, and that the regulation of transcripts is dynamic.

Overall these results suggest that the interactions between TGFbeta1 and testosterone regulate the growth and development of prostatic rudiments. The changes in phenotype of the VP induced by TGFbeta1 were not inhibited by the presence of testosterone. This suggests that the transcriptional regulation of *Tgfbeta* transcripts observed in these experiments may not be fully responsible for the changes observed in the architecture of the VP induced by TGFbeta1. However TGFbeta1 regulated both *Fgf10* and *Tgfbeta* transcript levels over seven hours in VPs, and the resultant changes may lead to the changes in growth and development observed in this thesis. In fact a preliminary study (where VPs were treated with TGFbeta1 for seven hours, TGFbeta1 was then removed and organs were further grown with or without testosterone for six days), suggested that short-term TGFbeta1 treatment of VPs would lead to the same changes in morphology

as observed over six days. However owing to limited time and technical problems associated with this approach this was not examined further. More research is needed to investigate this hypothesis.

8.4 Summary

In conclusion, it has been demonstrated that primary VMPC were a good, but not perfect, model to examine the regulation of *Fgf10* transcript levels. TGFbeta1 repressed *Fgf10* transcript levels in primary VMPC. The regulation was rapid and reversible, with maximum repression at three hours. TGFbeta1 was also shown to regulate *Fgf10* transcript levels in VMP but not in UR organ rudiments grown *in vitro*. TGFbeta1 also repressed *Fgf10* transcript levels in the VP, but the repression was to a much lesser extent than in the VMP, and primary VMPC and the effect was temporal. It has been proposed that the regulation of *Fgf10* transcript levels is strictly controlled by the presence of the epithelium in the VP, and that the presence of epithelial buds inhibited the TGFbeta1-mediated repression of *Fgf10* transcript levels. As the epithelium becomes more branched less repression of *Fgf10* transcript levels (in response to TGFbeta1) was observed suggesting that the epithelium was decreasing the effect of TGFbeta1. Regulation of *Fgf10* gene expression was also studied by the characterization of the *Fgf10* promoter. Using this method an Sp1 binding site was identified that, when deleted from the promoter, caused a decrease in promoter activity suggesting the Sp1 site was involved in *Fgf10* transcription. Also TGFbeta1 was found to decrease *Fgf10* promoter activity through the consensus Sp1 site, thus identifying a binding site in the *Fgf10* promoter that was mediating the effect of TGFbeta1.

It was demonstrated that *Tgfbeta1*, *Tgfbeta2*, and *Tgfbeta3* transcript expression correlated with growth and development of the prostate, indicating a potential role for TGFbeta isoforms in prostatic development. TGFbeta1 inhibited growth of the VP and

had differential effects on cell proliferation. Also TGFbeta1 and testosterone regulated *Tgfbeta* transcript levels in organ rudiments. The changes in phenotype of VP cultured with TGFbeta1 was not inhibited by testosterone, but the regulation of TGFbeta1-mediated transcriptional responses were inhibited by testosterone. This suggests that the TGFbeta1-mediated transcriptional changes examined in this thesis did not fully cause the changes in VP phenotype. However the short-term effects of TGFbeta1 on VPs might have caused a change in branching morphogenesis at an early stage of development that affected the patterning of growth. This suggests that the changes in *Fgf10* and *Tgfbeta* transcript levels might have in part been responsible for the changes in VP phenotype. Further research is needed to clarify this hypothesis.

Overall I have demonstrated that TGFbeta1 regulates *Fgf10* transcript levels in the prostate and that this effect is temporal and may be dependent on the presence of epithelium. It has also been demonstrated that *Tgfbeta* isoforms are expressed during periods of prostatic growth and may therefore play a role in prostate development. TGFbeta1 was shown to inhibit prostate growth. However although TGFbeta1 inhibited cell proliferation in the proximal region to urethra of the VP, surprisingly, TGFbeta1 stimulated cell proliferation in the distal region of the VP. As FGF10 promotes prostate growth and TGFbeta1 inhibits it, we can conclude that TGFbeta1 plays an antagonistic role to FGF10 during prostate development.

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